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(54) Title: USE OF RGM AND ITS MODULATORS

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(57) **Abstract:** The present invention relates to the use of a modulator of a polypeptide having or comprising an amino acid sequence as disclosed herein or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue, associated with seizures or associated with angiogenic disorders or disorders of the cardio-vascular system. Furthermore, the invention provides for the use of a modulator of a polypeptide having or comprising said amino acid sequence or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue, associated with angiogenic disorders or disorders of the cardio-vascular system. In addition the invention provides for the use of said polypeptide or said functional fragment or derivative thereof for the preparation of a pharmaceutical composition for preventing or treating tumor growth or formation of tumor metastases or as a marker of stem cells.

Use of RGM and its modulators

The present invention relates to the use of a modulator of a polypeptide having or comprising an amino acid sequence as disclosed herein or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue, associated with angiogenic disorders or disorders of the cardio-vascular system. Furthermore, the invention provides for the use of a modulator of a polypeptide having or comprising said amino acid sequence or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue, associated with seizures, associated with angiogenic disorders or disorders of the cardio-vascular system. In addition the invention provides for the use of said polypeptide or said functional fragment or derivative thereof for the preparation of a pharmaceutical composition for preventing or treating tumor growth or formation of tumor metastases or as a marker of stem cells.

Several documents are cited throughout the text of this specification. The disclosure content of each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference.

The most important mechanism in formation of embryonic nervous systems is the guidance of axons and growth cones by directional guidance cues (Goodman, Annu. Rev. Neurosci. 19 (1996), 341-77; Mueller, Annu. Rev. Neurosci 22, (1999),

351-88). A suitable model system for studying this guidance process is the retinotectal system of vertebrates. In the chick embryo approximately 2 million retinal ganglion cell (RGC) axons leave each eye and grow towards the contralateral tectum opticum to form a precise map (Mey & Thanos, (1992); J. Hirnforschung 33, 673-702). Having arrived at the anterior pole of the optic tectum, RGC axons start to invade their tectal target to find their target neurons. Mapping occurs in such a way, that RGC axons from nasal retina project to posterior tectum and temporal axons to anterior tectum. Along the dorso-ventral axis, axons coming from dorsal retina terminate in ventral tectum, whereas those from ventral retina end up in dorsal tectum. In the end a precise topographic map is formed, where neighborhood relationships in the retina are preserved in the tectum, so that axons from neighboring ganglion cells in the retina synapse with neighboring tectal neurons. Most important for formation of this map, are graded tectal guidance cues, read by retinal growth cones carrying corresponding receptors which also show a graded distribution (Sperry, Proc. Natl. Acad. Sci. USA 50 (1963), 703-710; Bonhoeffer & Gierer, Trends Neurosci. 7 (1984) 378-381). Position of each retinal growth cone in the tectal field is therefore determined by two sets of gradients: receptor gradients on ingrowing retinal axons and growth cones and ligand gradients on tectal cells (Gierer, Development 101 (1987), 479-489). The existence of the graded tectal ligands has been postulated from anatomical work, their identification however proved to be extremely difficult and was only made possible with the development of simple *in vitro* systems (Walter, Development 101 (1987), 685-96; Cox, Neuron 4 (1990), 31-7). In the stripe assay RGC axons grow on a membrane carpet, consisting of alternating lanes of anterior (a) and posterior (p) tectal membranes. On these carpets, temporal retinal axons grow on anterior tectal membranes and are repelled by the posterior lanes, whereas nasal axons do not distinguish between a and p membranes (Walter, Development 101 (1987), 685-96). The same specificity is also observed in the growth cone collapse assay (Raper & Kapfhammer, Neuron 4 (1990), 21-29), where temporal retinal growth cones collapse after addition of posterior tectal membrane vesicles but do not react to anterior tectal vesicles and where nasal growth cones are insensitive to either type of vesicles (Cox, (1990), loc. cit.). In both assay systems, treatment

of posterior tectal membranes with the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) which cleaves the lipid anchor of glycosylphosphatidylinositol (GPI)-linked proteins, removed their repellent and collapse-inducing activity (Walter, J. Physiol 84 (1990), 104-10).

One of the first repulsive guidance molecules identified in the retinotectal system of chick embryos was a GPI-anchored glycoprotein with a molecular weight of 33/35 kDa (Stahl, Neuron 5 (1990), 735-43). This 33/35 kDa molecule, later termed RGM (Repulsive Guidance Molecule), was active in both stripe and collapse-assays and was shown to be expressed in a low-anterior high-posterior gradient in the embryonic tecta of chick and rat (Mueller, Curr. Biol. 6 (1996), 1497-502; Mueller, Japan Scientific Societies Press (1997), 215-229). Due to the abnormal biochemical behaviour of RGM, the precise amino acid sequence was not obtainable. RGM was described as a molecule which is active during vertebrate development. Interestingly, RGM is down-regulated in the embryonic chick tectum after E12 and in the embryonic rat tectum after P2 and completely disappears after the embryonic stages (Müller (1992), Ph. D thesis University of Tübingen; Müller (1997) Japan Scientific Societies, 215-229) In 1996, Müller (loc. cit.) have shown that CALI (chromophore-assisted laser inactivation) of RGM eliminates the repulsive guidance activity of posterior tectal membranes /RGM. However, due to the presence of other guidance molecules, in particular of RAGS (repulsive axon guidance signal) and ELF-1 (Eph ligand family 1), a complete elimination of guidance was not always detected and it was speculated that RGM acts in concert with RAGS (now termed ephrin-A5) and ELF-1 (ephrin-A2). It was furthermore envisaged that RGM may be a co-factor potentiating the activity of RAGS and ELF-1 in embryonic guidance events.

In 1980/81 the group of Aguayo found that, when peripheral neurons are transplanted/grafted into injured CNS of adult, axon growth of CNS neurons is induced (David, Science 214 (1981), 931-933). Therefore, it was speculated that CNS neurons have still the ability and capacity of neurite-outgrowth and/or regeneration, if a suitable environment would be provided. Furthermore, it was speculated that "CNS-neuron regeneration inhibitors" may exist.

In 1988, Caroni and Schwab (Neuron 1, 85-96) described two inhibitors of 35 kDa and 250 kDa , isolated from rat CNS myelin (NI-35 and NI-250; see also Schnell, Nature 343 (1990) 269-272; Caroni, J.Cell Biol. 106 (1988), 1291-1288).

In 2000, the DNA encoding for NI-220/250 was deduced and the corresponding potent inhibitor of neurite growth was termed Nogo-A (Chen, Nature 403 (2000), 434-438. The membrane-bound Nogo turned out to be a member of the reticulon family (GrandPré, Nature 403 (2000), 439-444).

Further factors which mediate neuronal outgrowth inhibition have first been isolated in grasshoppers, and termed „fasciclin IV“ and later „collapsin“ in chicken. These inhibitors belong to the so-called semaphorin family. Semaphorins have been reported in a wide range of species and described as transmembrane proteins (see, inter alia, Kolodkin Cell 75 (1993) 1389-99, Püschel, Neuron 14 (1995), 941-948). Yet, it was also shown that not all semaphorins have inhibitory activity. Some members of said family, e.g. semaphorin E, act as an attractive guidance signal for cortical axons (Bagnard, Development 125 (1998), 5043-5053).

A further system of repulsive guidance molecules is the ephrin-Eph system. Ephrins are ligands of the Eph receptor kinases and are implicated as positional labels that may guide the development of neural topographic maps (Flanagan, Ann. Rev. Neurosci. 21 (1998), 309-345). Ephrins are grouped in two classes, the A-ephrins which are linked to the membrane by a glycosylphosphatidylinositol-anchor (GPI-anchor) and the B-ephrins carrying a transmembrane domain (Eph nomenclature committee 1997). Two members of the A-ephrins, ephrin-A2 and ephrin-A5, expressed in low anterior – high posterior gradients in the optic tectum, have recently been shown to be involved in repulsive guidance of retinal ganglion cell axons in vitro and in vivo (see, inter alia (Drescher, Cell 82 (1995), 359-70; Cheng, Cell 79 (1994), 157-168; Feldheim, Neuron 21 (1998), 563-74; Feldheim, Neuron 25 (2000), 563-74)

Considering the fact that a plurality of physiological disorders or injuries are related to altered cellular migration processes, the technical problems underlying the present invention was to provide for means and methods for modifying altered developmental or cellular (migration) processes which lead to disease conditions.

Accordingly, the present invention relates to the use of an modulator of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs.18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue, associated with angiogenic disorders or disorders of the cardio-vascular system and associated with tumor formation and tumor growth.

In context of the present invention, and as documented in the appended examples, it was surprisingly found that the repulsive guidance molecule (RGM) is not only expressed during vertebrate development but is re-expressed in adult tissue, in particular in damaged adult tissues. It was, *inter alia*, surprisingly found that RGM is re-expressed after damage of the nervous tissue, after traumatic events or focal ischemias. The present invention provides for the complete nucleotide sequence and/or amino acid of RGM (see, e.g. SEQ ID NO: 17 or 18 depicting the RGM sequence of chicken or SEQ ID NO: 20 to 25 depicting the human RGM homologues.) RGM, as pointed out herein above, is a glycoprotein, linked to membranes by a GPI-anchor. Said GPI-anchor also carries a cross-reacting determinant (CRD) epitope and its carbohydrate part is able to bind peanut lectin. As documented herein, the RGM protein is a potent growth inhibitor and can assert neurite growth inhibition in picomolar concentrations.

The term "modulator" as employed herein relates to "inhibitors" as well as "activators" of RGM function. Most preferably said "modulation" is an inhibition, wherein said inhibition may be a partial or a complete inhibition.

The term „amino acid sequence of SEQ ID NO: 18, 20, 23 or 25 as employed herein relates to the amino acid sequence of RGM (repulsive guidance molecule) and relates to the RGM polypeptide of chicken or human, respectively. In particular, SEQ ID NOs: 20 and 21 depict human RGM1. Human RGM1 has been localized on chromosome 15. Further, human RGMs comprise RGM2 and RGM3. RGM2 is depicted in SEQ ID

NO: 23 (amino acid sequence) and is encoded by a nucleotide sequence as shown in SEQ ID NO: 22. Human RGM2 has been localized on chromosome 5. Furthermore, human RGM3 is shown in appended SEQ ID NO: 25 (amino acid sequence) and encoded by a nucleotide sequence as depicted in SEQ ID NO: 24. Human RGM3 is located on chromosome 1. Yet, as will be discussed herein below, said term relates also to further RGM homologues.

The term "(poly)peptide" means, in accordance with the present invention, a peptide, a protein, or a (poly)peptide which encompasses amino acid chains of a given length, wherein the amino acid residues are linked by covalent peptide bonds. However, peptidomimetics of such RGM proteins/(poly)peptides wherein amino acid(s) and/or peptide bond(s) have been replaced by functional analogs are also encompassed by the invention.

The present invention is not restricted to RGM from human, mouse or chicken and its inhibitors but also relates to the use of inhibitors of RGM or of RGM itself (or functional fragments or derivatives thereof) from other species. Since the present invention provides for the use of amino acid sequences/polypeptides of RGM and its corresponding inhibitors and since the amino acid sequences of human and chicken RGM are disclosed herein, the person skilled in the art is provided with the information to obtain RGM sequences from other species, like, inter alia, mouse, rat, pig, etc. The relevant methods are known in the art and may be carried out by standard methods, employing, inter alia, degenerate and non degenerate primers in PCR-techniques. Such molecular biology methods are well known in the art and, e.g., described in Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)) and Ausubel, "Current Protocols in Molecular Biology", Green Publishing Associates; and Wiley Interscience, N.Y. (1989).

Furthermore, as employed in the context of the present invention, the term "RGM", "RGM modulator" and "RGM-inhibitor" also relates to RGM molecules (and their corresponding inhibitors) which are variants or homologs of the RGM molecules (and their inhibitors) as described herein. "Homology" in this context is understood to refer in this context to a sequence identity of RGMs of at least 70%, preferably more than 80%

and still more preferably more than 90% on the amino acid level. The present invention, however, comprises also (poly)peptides deviating from wildtype amino acid sequences of human or chicken RGMs described herein, wherein said deviation may be, for example, the result of amino acid and/or nucleotide substitution(s), deletion(s), addition(s), insertion(s), duplication(s), inversion(s) and/or recombination(s) either alone or in combination. Those deviations may naturally occur or be produced via recombinant DNA techniques well known in the art. The term "variation" as employed herein also comprises "allelic variants". These allelic variations may be naturally occurring allelic variants, splice variants as well as synthetically produced or genetically engineered variants.

The term "polynucleotide" in accordance with the present invention comprises coding and, wherever applicable, non-coding sequences (like promotors, enhancers etc.). It comprises DNA, RNA as well as PNA. In accordance with the present invention, the term "polynucleotide/nucleic acid molecule" comprises also any feasible derivative of a nucleic acid to which a nucleic acid probe may hybridize. Said nucleic acid probe itself may be a derivative of a nucleic acid molecule capable of hybridizing to said nucleic acid molecule or said derivative thereof. The term "nucleic acid molecule" further comprises peptide nucleic acids (PNAs) containing DNA analogs with amide backbone linkages (Nielsen, Science 254 (1991), 1497-1500). The term "nucleic acid molecule" which encodes a RGM (poly)peptide or a functional fragment/derivative thereof, in connection with the present invention, is defined either by (a) the specific nucleic acid sequences encoding said (poly)peptide specified in the present invention or (b) by nucleic acid sequences hybridizing under stringent conditions to the complementary strand of the nucleotide sequences of (a) and encoding a (poly)peptide deviating from the nucleic acid of (a) by one or more nucleotide substitutions, deletions, additions or inversions and wherein the nucleotide sequence shows at least 70%, more preferably at least 80% identity with the nucleotide sequence of said encoded RGM (poly)peptide having an amino acid sequence as defined herein above and functions as a RGM (or a functional fragment/derivative thereof).

The term "modulator" as employed herein also comprises the term "inhibitor", as mentioned herein above.

The term "inhibitor of a polypeptide having or comprising the amino acid sequence of SEQ ID NOS 18, 20, 23 or 25 or a functional fragment or derivative thereof", therefore, not only relates to the specific inhibitors of human or chicken RGM but also relates to inhibitors of RGM (or functional fragments or derivatives thereof) of other species. Useful inhibitors are disclosed herein as well as described herein below and in the appended examples.

The term "inhibitor" also comprises "modulators" of the RGM polypeptides and/or the RGM encoding nucleic acid molecule/gene. In context of this invention it is also envisaged that said "modulation" leads, when desired, to an activation of RGM.

The term "functional fragment or derivative thereof" in context of the present invention and in relation to the herein described RGM molecules comprises fragments of the RGM molecules defined herein having a length of at least 25, more preferably at least 50, more preferably at least 75, even more preferably at least 100 amino acids. Functional fragments of the herein identified RGM molecules or RGM molecules of other species (homologous RGMs) may be comprised in fusion and/or chimeric proteins. "Functional fragments" comprise RGM fragments (or its encoding nucleic acid molecules) which are able to replace RGM full length molecules in corresponding assays (as disclosed herein in the appended examples, e.g. collapse and/or stripe assays) or may elucidate an anti-RGM specific immune-response and/or lead to specific anti-RGM antibodies. An example of such a "functional fragment" is, inter alia, the functional fragment of chicken RGM depicted in SEQ ID NO: 19. In context of the present invention, polynucleotides encoding functional fragments of RGM and/or its derivatives have preferably at least 15, more preferably at least 30, more preferably at least 90, more preferably of at least 150, more preferably of at least 300 nucleotides. The term "derivative" means in context of their invention derivatives of RGM molecules and/or their encoding nucleic acid molecules and refer to natural derivatives (like allelic variants) as well as recombinantly produced derivatives/variants which may differ from

the herein described RGM molecules by at least one modification/mutation, e.g. at least one deletion, substitution, addition, inversion or duplication. The term "derivative" also comprises chemical modifications. The term "derivative" as employed herein in context of the RGM molecule also comprises soluble RGM molecules which do not comprise any membrane anchorage.

As mentioned herein above, the present invention provides for the use of a modulator, preferably an inhibitor, of RGM molecules and/or their corresponding encoding polynucleotides/nucleic acid molecules for the preparation of a pharmaceutical composition for preventing, alleviating or treating various disorders of the nervous system, angiogenic disorders or disorders of the cardio-vascular system and malignancies of different etiology.

In a preferred embodiment, said disorders of the nervous system comprise degeneration or injury of vertebrate nervous tissue, in particular neurodegenerative diseases, nerve fiber injuries and disorders related to nerve fiber losses.

Said neurodegenerative diseases may be selected from the group consisting of motorneuronal diseases (MND), amyotrophic lateral sclerosis (ALS), Alzheimers disease, Parkinsons disease, progressive bulbar palsy, progressive muscular atrophy, HIV-related dementia and spinal muscular atrophy(ies), Down's Syndrome, Huntington's Disease, Creutzfeldt-Jacob Disease, Gerstmann-Sträussler Syndrome, kuru, Scrapie, transmissible mink encephalopathy, other unknown prion diseases, multiple system atrophy, Riley-Day familial dysautonomia said nerve fiber injuries may be selected from the group consisting of spinal cord injury(ies), brain injuries related to raised intracranial pressure, trauma, secondary damage due to increased intracranial pressure, infection, infarction, exposure to toxic agents, malignancy and paraneoplastic syndromes and wherein said disorders related to nerve fiber losses may be selected from the group consisting of paresis of nervus facialis, nervus medianus, nervus ulnaris, nervus axillaris, nervus thoracicus longus, nervus radialis and for of other peripheral nerves, and other aquired and non-aquired deseases of the (human) central and peripheral nervous system.

The above mentioned spinal cord and brain injuries not only comprise traumatic injuries but also relate to injuries caused by stroke, ischemia and the like. It is in particular envisaged that the inhibitors as defined herein below and comprising, inter alia, anti-RGM antibodies be employed in the medical art to stimulate nerve fiber growth in individuals, in particular in vertebrates, most preferably in humans.

In a more preferred embodiment of the present invention, the invention provides for the use of a modulator, preferably an inhibitor to RGM (or a functional fragment or derivative thereof) for the preparation of a pharmaceutical composition for the treatment of disorders of the cardio-vascular system, wherein these disorders, e.g., comprise disorders of the blood-brain barrier, brain oedema, secondary brain damages due to increased intracranial pressure, infection, infarction, ischemia, hypoxia, hypoglycemia, exposure to toxic agents, malignancy, paraneoplastic syndromes.

It is envisaged, without being bound by theory, that RGM inhibitors may stimulate surviving neurons to project collateral fibers into the diseased tissue, e.g. the ischemic tissue.

As illustrated in the appended examples, RGM is expressed locally at the site of artificial transection of brain/spinal cord tissue in test animals (like rats), e.g., in the penumbra region surrounding an ischemic core of a human suffering focal ischemia in the temporal cortex. Furthermore, it is documented in the appended examples that RGM is, surprisingly, expressed in tissue(s) having experienced from traumatic brain injuries. The invention also relates to the use of a RGM polypeptide or a functional fragment or derivative thereof or the use of a polynucleotide encoding the same (polypeptides and polynucleotides as defined herein), wherein the above described disease or condition associated with seizures is epilepsy. An epilepsy is thereby characterized by an epileptic seizure as a convulsion or transient abnormal event experienced by the subject, e.g. a human patient, due to a paroxysmal discharge of (cerebral) neurons. The epileptic seizures comprise tonic seizures, tonic-clonic

seizures (grand mal), myoclonic seizures, absence seizures as well as akinetic seizures. Yet, also comprised are in context of this invention simple partial seizures, e.g. Jacksonian seizures and seizures due to perinatal trauma and/or fetal anoxia. As mentioned herein below, the uses described herein relate in particular to the preparation of pharmaceutical compositions for the treatment of diseases/conditions associated with aberrant sprouting of nerve fibres, like epilepsy; see also Routbort, Neuroscience 94 (1999), 755-765.

In an even more preferred embodiment of the invention, the modulator, preferably the inhibitor of RGM (or of its functional fragment or derivative thereof or of its encoding nucleic acid molecule) is used for the preparation of a pharmaceutical composition for the modification of neovascularization. Said modification may comprise activation as well as stimulation. It is in particular envisaged that said neovascularisation be stimulated and/or activated in diseased tissue, like inter alia, ischemic and/or infarctious tissue. Furthermore, it is envisaged that the RGM-inhibitors described herein may be employed in the regulation of the blood-brain barrier permeability.

It is furthermore envisaged that said modulators, preferably said inhibitors for RGM be employed in the alleviation, prevention and/or inhibition of progression of vascular plaque formation (e.g. atherosclerosis) in cardio-vascular, cerebo-vascular and/or nephrovascular diseases/disorders.

Furthermore, the present invention provides for the use of a modulator, preferably an inhibitor of RGM as defined herein for the preparation of a pharmaceutical composition for remyelination. Therefore, the present invention provides for a pharmaceutical composition for the treatment of demyelinating diseases of the CNS, like multiple sclerosis or of demyelinating diseases like peripheral neuropathy caused by diphtheria toxin, Landry-Guillain-Barré-Syndrom, Elsberg-Syndrom, Charcot-Marie-Tooth disease and other polyneuropaties. A particular preferred inhibitor of RGM in this context is an antibody directed against RGM, e.g. an IgM antibody. It has previously been shown that certain IgMs bind to oligodendrocytes and thereby induce remyelination. IgM

antibodies against RGM are known in the art and comprise e.g. the F3D4 described in the appended examples.

In addition the invention provides for the use of a RGM polypeptide as defined herein or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the activity of autoreactive immune cells or with overactive inflammatory cells. Most preferably these cells are T-cells.

Furthermore, the present invention relates to the use of a modulator, preferably an inhibitor or another RGM binding molecule of a RGM polypeptide or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or of fragment/derivative thereof for modifying and/or altering the differentiation status of neuronal stem cells and/or their progenitors. Said stem cells are normally found in the subventricular zones of many brain regions. It is known that factors in the microenvironment of the brain dramatically influence the differentiation of undifferentiated stem cells. It is assumed that due to the characteristic expression of RGM in the subventricular layers of many different brain regions, this molecule could be a marker for stem cells. Furthermore, RGM inhibitors, like antibodies could be useful markers for stem cells. Most important in stem cell biology is the understanding of factors influencing their differentiation. It is therefore assumed that RGM inhibitors change the developmental fate of these cells.

As documented in the appended examples, RGM is not only expressed in ischemic tissue but is also expressed in scar tissue surrounding (brain) lesions.

It is particularly preferred that the modulator, preferably the inhibitor of the RGM molecule (or its functional fragment or derivative) is an antibody or a fragment or a derivative thereof, is an aptamer, is a specific receptor molecule capable of interacting with a RGM polypeptide or with a functional fragment or derivative thereof, or is a

specific nucleic acid molecule interacting with a polynucleotide encoding an RGM and/or the polypeptide as defined herein.

The antibody to be used in context of the present invention can be, for example, polyclonal or monoclonal antibodies. Techniques for the production of antibodies are well known in the art and described, e.g. in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. The production of specific anti-RGM antibodies is further known in the art (see, e.g. Müller (1996) loc.cit.) or described in the appended examples.

The term "antibody" as employed herein also comprises chimeric, single chain and humanized antibodies, as well as antibody fragments, like, inter alia, Fab fragments. Antibody fragments or derivatives further comprise F(ab')₂, Fv or scFv fragments; see, for example, Harlow and Lane, loc.cit.. Various procedures are known in the art and may be used for the production of such antibodies and/or fragments, see also appended examples. Thus, the (antibody) derivatives can be produced by peptidomimetics. Further, techniques described for the production of single chain antibodies (see, inter alia, US Patent 4,946,778) can be adapted to produce single chain antibodies to polypeptide(s) of this invention. Also, transgenic animals may be used to express humanized antibodies to polypeptides of this invention. Most preferably, the antibody to be used in the invention is a monoclonal antibody, for example the F3D4 antibody described in the appended examples may be employed when an IgM is desired. The general methodology for producing, monoclonal antibodies is well-known and has been described in, for example, Köhler and Milstein, Nature 256 (1975), 494-496 and reviewed in J.G.R. Hurrel, ed., "Monoclonal Hybridoma Antibodies: Techniques and Applications", CRC Press Inc., Boca Raton, FL (1982), as well as that taught by L. T. Mimms et al., Virology 176 (1990), 604-619.

Preferably, said antibodies (or inhibitors) are directed against functional fragments of the RGM polypeptide. As pointed out herein above and as documented in the appended examples, such functional fragments are easily deducible for the person skilled in the art and, correspondingly, relevant antibodies (or other inhibitors) may be produced.

The "modulator", preferably the "inhibitor" as defined herein may also be an aptamer. In the context of the present invention, the term "aptamer" comprises nucleic acids such as RNA, ssDNA (ss = single stranded), modified RNA, modified ssDNA or PNAs which bind a plurality of target sequences having a high specificity and affinity. Aptamers are well known in the art and, inter alia, described in Famulok, Curr. Op. Chem. Biol. 2 (1998), 320-327. The preparation of aptamers is well known in the art and may involve, inter alia, the use of combinatorial RNA libraries to identify binding sites (Gold, Ann. Rev. Biochem. 64 (1995), 763-797). Said other receptors may, for example, be derived from said antibody etc. by peptidomimetics.

Other specific "receptor" molecules which may function as inhibitors of the RGM polypeptides are also comprised in this invention. Said specific receptors may be deduced by methods known in the art and comprise binding assays and/or interaction assays. These may, inter alia, involve assays in the ELISA-format or FRET-format. Said "inhibitor" may also comprise specific peptides binding to and/or interfering with RGM.

Furthermore, the above recited "modulator", preferably "inhibitor" may function at the level of RGM gene expression. Therefore, the inhibitor may be a (specific) nucleic acid molecule interacting with a polynucleotide encoding a RGM molecule (or a functional fragment or derivative thereof.) These inhibitors may, e.g., comprise antisense nucleic acid molecules or ribozymes.

The nucleic acid molecule encoding RGM (and as disclosed herein, e.g., SEQ ID NO: 17) may be employed to construct appropriate anti-sense oligonucleotides. Said anti-sense oligonucleotides are able to inhibit the function of wild-type (or mutant) RGM genes and comprise, preferably, at least 15 nucleotides, more preferably at least 20 nucleotides, even more preferably 30 nucleotides and most preferably at least 40 nucleotides.

In addition, ribozyme approaches are also envisaged for use in this invention. Ribozymes may specifically cleave the nucleic acid molecule encoding RGMs.

In the context of the present invention ribozymes comprise, inter alia, hammerhead ribozymes, hammerhead ribozymes with altered core sequences or deoxyribozymes (see, e.g., Santoro, Proc. Natl. Acad. Sci. USA 94 (1997), 4262) and may comprise natural and in vitro selected and/or synthesized ribozymes.

Nucleic acid molecules according to the present invention which are complementary to nucleic acid molecules coding for proteins/(poly)peptides regulating, causing or contributing to obesity and/or encoding a mammalian (poly)peptide involved in the regulation of body weight (see herein below) may be used for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave nucleic acid molecules of the invention. Selection of the appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith, eds. Academic Press, Inc. (1995), 449-460.

Said "inhibitor" may also comprise double-stranded RNAs, which lead to RNA-mediated gene interference (see Sharp, Genes and Dev. 13 (1999), 139-141)

Further potential inhibitors of RGM may be found and/or deduced by interaction assay and employing corresponding read-out systems. These are known in the art and comprise, inter alia, two hybrid screenings (as, described, inter alia, in EP-0 963 376, WO 98/25947, WO 00/02911) GST-pull-down columns, co-precipitation assays from cell extracts as described, inter alia, in Kasus-Jacobi, Oncogene 19 (2000), 2052-2059, "interaction-trap" systems (as described, inter alia, in US 6,004,746) expression cloning (e.g. lambda gt11), phage display (as described, inter alia, in US 5,541,109), in vitro binding assays and the like. Further interaction assay methods and corresponding read out systems are, inter alia, described in US 5,525,490, WO 99/51741, WO 00/17221, WO 00/14271 or WO 00/05410.

A further objective of the present invention is to provide for the use of a RGM polypeptide and/or of polypeptide having or comprising the amino acid sequence of SEQ ID NOS. 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a

polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with excessive collateral sprouting of nerve fibres.

The present invention, therefore, provides for the medical use of RGM protein(s) and/or functional fragments/derivatives thereof or for the use of polynucleotides encoding said RGM protein(s) in conditions where excessive collateral sprouting occurs. Said conditions comprise, but are not limited to, epilepsy, phantom pain and neuropathic pain. For example, McNamara (Nat. Suppl. 399 (1999), A15-A22) has described that said sprouting occurs in certain types of epilepsy. The RGM molecule, either naturally isolated or recombinantly produced, or its functional fragments/derivatives may therefore be employed as potent "stop" signals for growing nerve fibres. The feasibility of such an approach has been shown by Tanelian (Nat. Med. 3 (1997), 1398-1401) who employed a semaphorin for inhibition of nerve fiber growth.

In yet another embodiment, the present invention provides for the use of RGM and/or of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing or treating tumor growth or formation of tumor metastases.

RGM (naturally isolated or recombinantly produced) and/or functional fragments thereof may be employed for the preparation of a pharmaceutical composition for the treatment of neoplastic disorders, in particular of disorders related to tumor (cell) migration, metastasis and/or tumor invasion. Furthermore, it is envisaged that RGM inhibits undesired neovascularisation. Said neovascularisation, as an angiogenic disorder during neoplastic events, should be prevented in order to limit, inter alia, tumor growth.

Growth cones of neurons and (invasive) tumor cells secrets a cocktail of proteases (uPA, tPA, MNPs, etc.) in order to degrade extracellular matrix. Furthermore, similar mechanisms for adhesion and (cell) migration are employed by these cellular systems.

RGM and/or its functional fragments may be employed to actively stimulate withdrawal of lamellipodia of tumor cells and/or to induce their collapse. As demonstrated in the appended examples RGM also influences tumor growth behaviour, i.e. is capable of negatively influencing tumor growth.

In addition the invention provides for the use of a RGM polypeptide as defined herein or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the activity of autoreactive immune cells or with overactive inflammatory cells. Most preferably these cells are T-cells.

In yet another embodiment, the invention provides for the use of a RGM polypeptide having or comprising, inter alia, the amino acid sequence of SEQ ID NOs.18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for the treatment of inflammation processes and/or allergies, for wound healing or for the suppression/alleviation of scar formation. Scar tissue is formed by invading cells, most importantly by fibroblasts and/or glial cells. Migration and adhesion of these cells are required to get to the lesion side. RGM or an active fragment/derivative could prevent accumulation of these cells in the lesion side, thereby preventing or slowing down scar formation. In inflammatory reactions cells migrate to the inflamed region and RGM or its active fragment/derivative prevent or reduce migration of these cells to the side of inflammation, thereby preventing overactive inflammatory reactions.

In context of the present invention, the term "pharmaceutical composition" also comprises optionally further comprising an acceptable carrier and/or diluent and/or excipient. The pharmaceutical composition of the present invention may be particularly useful in preventing and/or treating pathological disorders in vertebrates, like humans. Said pathological disorders comprise, but are not limited to, neurological, neurodegenerative and/or neoplastic disorders as well

as disorders associated with seizures, e.g. epilepsy. These disorders comprise, inter alia, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (FALS/SALS), ischemia, stroke, epilepsy, AIDS dementia and cancer. The pharmaceutical composition may also be used for prophylactic purposes. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. However, it is also envisaged that the pharmaceutical compositions are directly applied to the nervous tissue. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, general health, age, sex, the particular compound to be administered, time and route of administration, and other drugs being administered concurrently. Pharmaceutically active matter may be present preferably, inter alia, in amounts between 1 ng and 1000 mg per dose, more preferably in amounts of 1 ng to 100 mg however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously. The compositions of the invention may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered

media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents, depending on the intended use of the pharmaceutical composition. Such agents may be drugs acting on the central nervous system as well as on small, unmyelinated sensory nerve terminals (like in the skin), neurons of the peripheral nervous system of the digestive tract., etc.

It is also understood that the pharmaceutical composition as defined herein may comprise nucleic acid molecules encoding RGMs (and/or functional fragments or derivatives thereof) or corresponding RGM inhibitors or defined herein. As mentioned herein-above, said inhibitors comprise, but are not limited to, antibodies, aptamer, RGM-interacting peptides as well as inhibitors interacting with the RGM-encoding polynucleotides.

Accordingly, the present invention also provides for a method of treating, preventing and/or alleviating pathological disorders and conditions as defined herein, whereby said method comprises administering to a subject in need of such a treatment a pharmaceutical composition/medicament as defined herein. Preferably, said subject is a human.

The nucleic acid molecules may be particularly useful in gene therapy approaches and may comprise DNA, RNA as well as PNA. Said nucleic acid molecules may be comprised in suitable vectors, either *inter alia*, gene expression vectors. Such a vector may be, e.g., a plasmid, cosmid, virus, bacteriophage or another vector used e.g. conventionally in genetic engineering, and may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions.

Furthermore, the vectors may, in addition to the nucleic acid sequences encoding RGM or its corresponding inhibitors, comprise expression control elements, allowing proper expression of the coding regions in suitable host cells or tissues. Such control elements are known to the artisan and may include a promoter, translation initiation codon, translation and insertion site for introducing an insert into the vector. Preferably, the nucleic acid molecule of the invention is operatively linked to said expression control sequences allowing expression in (eukaryotic) cells. Particularly preferred are in this context control sequences which allow for correct expression in neuronal cells and/or cells derived from nervous tissue.

Control elements ensuring expression in eukaryotic cells are well known to those skilled in the art. As mentioned above, they usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression in for example mammalian host cells comprise the CMV- HSV thymidine kinase promoter, SV40, RSV-promoter (Rous sarcoma virus), human elongation factor 1 α -promoter, CMV enhancer, CaM-kinase promoter or SV40-enhancer. For the expression for example in nervous tissue and/or cells derived therefrom, several regulatory sequences are well known in the art, like the minimal promoter sequence of human neurofilament L (Charron, J. Biol. Chem 270 (1995), 25739-25745). Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pRc/CMV, pcDNA1, pcDNA3 (In-Vitrogen, as used, inter alia in the appended examples), pSPORT1 (GIBCO BRL) or pGEMHE (Promega). Beside the nucleic acid molecules defined herein, the vector may further comprise nucleic acid sequences encoding for secretion signals. Such sequences are well known to the person skilled in the art. Furthermore, depending on the expression system

used leader sequences capable of directing the protein/(poly)peptide to a cellular compartment may be added to the coding sequence of the nucleic acid molecules of the invention and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a part thereof,

As mentioned herein above, said vector may also be, besides an expression vector, a gene transfer and/or gene targeting vector. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors, vector systems and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813, Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang, Nature Medicine 2 (1996), 714-716; WO 94/29469; WO 97/00957, Schaper, Current Opinion in Biotechnology 7 (1996), 635-640 Verma, Nature 389 (1997), 239-242 WO 94/29469, WO 97/00957, US 5,580,859, US 589,66 or US 4,394,448 and references cited therein.

In particular, said vectors and/or gene delivery systems are also described in gene therapy approaches in neurological tissue/cells (see, inter alia Blömer, J. Virology 71 (1997) 6641-6649) or in the hypothalamus (see, inter alia, Geddes, Front Neuroendocrinol. 20 (1999), 296-316 or Geddes, Nat. Med. 3 (1997), 1402-1404). Further suitable gene therapy constructs for use in neurological cells/tissues are known in the art, for example in Meier (1999), J. Neuropathol. Exp. Neurol. 58, 1099-1110. The nucleic acid molecules and vectors of the invention may be designed for direct introduction or for introduction via liposomes, viral vectors (e.g. adenoviral, retroviral), electroporation, ballistic (e.g. gene gun) or other delivery systems into the cell. Additionally, a baculoviral system can be used as eukaryotic expression system for the nucleic acid molecules described herein.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of partially or completely curing a disease and/or adverse effect attributed to the disease. The term "treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e. arresting its development; or (c) relieving the disease, i.e. causing regression of the disease.

In yet another embodiment, the present invention provides for the use of a (RGM) polypeptide and/or a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative as a marker of stem cells. Since it is envisaged that stem cells as well as their undifferentiated progenitor cells express RGM, RGM and/or functional fragments or derivatives thereof may be employed to influence the differentiation/differentiation pattern of said stem cells.

It is furthermore envisaged that antibodies directed against RGMs, in particular directed against polypeptides disclosed herein or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 (or (a) functional fragment(s)/derivative(s) thereof) may be employed to influence the differentiation of (neuronal) stem cells and (neuronal) progenitor cells. It is particularly preferred that said antibodies (as well as other RGM-inhibitors and/or RGM-binding molecules) be employed to selectively label stem cells. Therefore these reagents may be employed as markers for stem cells. It is also envisaged that peptides or derivatives be employed in said purpose.

In a particularly preferred embodiment of the present invention, the polypeptide and/or fragment thereof which comprises or has an amino acid sequence as depicted in SEQ

ID NOs 18, 20, 23 or 25 and/or is a RGM molecule to be used in accordance with their invention is a soluble, i.e. not membrane bound molecule.

As shown in Davis (1994), Science 266, 816-819 ephrins, in particular A-ephrins, are not active in soluble, monomeric form. In contrast, soluble RGMs are active and may function without any membrane-attachment. RGM, in contrast to ephrins, is capable of self-formation of dimers and/or of the formation of higher aggregates. The invention also provides for the use of a RGM molecule and/or a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or a fragment or a derivative for the preparation of a pharmaceutical composition for alleviating, preventing and/or treating homeostatic and/or bleeding disorders and/or vascular damage.

It is envisaged, without being bound by theory, that RGMs may, due to their structural homology to von-Willebrand factor (vWF), be employed in the treatment of said disorders/diseases. Furthermore, it is envisaged that RGM may interact with von-Willebrand factor and that said molecule, thereby, influences the activity of vWF. Furthermore, the inhibitors as defined herein should be employed in disorders where immune cells invade the brain, like multiple sclerosis, encephalomyelitis disseminata.

The present invention also provides for the use of an antibody or a fragment or a derivative thereof, or an aptamer, or a binding molecule capable of interacting with a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or with functional fragment or derivative thereof or of an nucleic acid molecule capable of interacting with a polynucleotide encoding said polypeptide or a fragment thereof for the preparation of a diagnostic composition for detecting neurological and/or neurodegenerative disorders or dispositions thereto.

The diagnostic composition may be used, inter alia, for methods for determining the expression of the nucleic acids encoding RGM polypeptides by detecting, inter alia, the presence of the corresponding mRNA which comprises isolation of RNA from a cell, contacting the RNA so obtained with a nucleic acid probe as described above under hybridizing conditions, and detecting the presence of mRNAs hybridized to the probe.

Furthermore, corresponding mutations and/or alterations may be detected. Furthermore, RGM (poly)peptides can be detected with methods known in the art , which comprise, inter alia, immunological methods, like, ELISA or Western blotting.

The diagnostic composition of the invention may be useful, inter alia, in detecting the prevalence, the onset or the progress of a disease related to the aberrant expression of a RGM polypeptide. Accordingly, the diagnostic composition of the invention may be used, inter alia, for assessing the prevalence, the onset and/or the disease status of neurological, neurodegenerative and/or inflammatory disorders, as defined herein above. It is also contemplated that anti-RGM antibodies, aptamers etc. and compositions comprising such antibodies, aptamers, etc. may be useful in discriminating (the) stage(s) of a disease.

The diagnostic composition optionally comprises suitable means for detection. The nucleic acid molecule(s), vector(s), antibody(ies), (poly)peptide(s), described above are, for example, suitable for use in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for the purposes of the invention.

Solid phase carriers are known to those in the art and may comprise polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass and/or silicon chips and surfaces, nitrocellulose strips, membranes, sheets, duracytes and the walls of wells of a reaction tray, plastic tubes or other test tubes. Suitable methods of immobilizing nucleic acid molecule(s), vector(s), host(s), antibody(ies), (poly)peptide(s), fusion protein(s) etc. on solid phases include but are not limited to ionic, hydrophobic, covalent interactions and the like. Examples of immunoassays which can utilize said compounds of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Commonly used detection assays can comprise radioisotopic or non-radioisotopic methods. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich

(immunometric assay) and the Northern or Southern blot assay. Furthermore, these detection methods comprise, inter alia, IRMA (Immune Radioimmunometric Assay), EIA (Enzyme Immuno Assay), ELISA (Enzyme Linked Immuno Assay), FIA (Fluorescent Immuno Assay), and CLIA (Chemioluminescent Immune Assay). Furthermore, the diagnostic compounds of the present invention may be employed in techniques like FRET (Fluorescence Resonance Energy Transfer) assays.

The nucleic acid sequences encoding RGMs of other species as well as variants of RGMs are easily deducible from the information provided herein. These nucleic acid sequences are particularly useful, as pointed out herein above, in medical and/or diagnostic setting, but they also provide for important research tools. These tools may be employed, inter alia, for the generation of transgenic animals which overexpress or suppress RGMs or wherein the RGM gene is silenced and/or deleted. Furthermore, said sequences may be employed to detect and/or elucidate RGM interaction partners and/or molecules binding to and/or interfering with RGMs.

The Figures show:

Figure 1: RGM protein fractions induce collapse of RGC growth cones.

Solubilized membrane proteins from E9/E10 chick brains were loaded on two different ion exchange columns, a DEAE anion exchange column and a cation exchange column. RGM was eluted from the cation exchange column at a NaCl concentration of 200 - 400 mM in two 1 ml fractions (4 + 5) was incorporated into lecithin vesicles and lecithin vesicles were used in collapse experiments with RGC growth cones. RGM-containing fractions (4 + 5, arrows), but not RGM-free fractions induced extensive collapse (>90%) of RGC growth cones. Neither ephrin-A5 nor ephrin-A2 could be detected with specific antibodies, in RGM - fractions. RGC axons and growth cones on laminin were stained with Alexa-Phalloidin. Western blots from two dimensional gels were incubated with the F3D4 monoclonal antibody, and were subsequently stained by a whole protein, india ink stain.

Figure 2: Comparative two dimensional gel analysis of tectal proteins and RGM sequences.

A: Membranes from E9/10 anterior and posterior chick tecta were enriched and treated with buffer (C) or with PI-PLC (E), to remove GPI-anchored proteins. The putative RGM (arrow in Anterior-E + Posterior-E), a PI-PLC cleavable basic protein with a molecular weight of 33 kDa, was cut out and was used for nanoelectrospray tandem mass spectrometry. Two dimensional gels were stained with silver. No anterior-posterior difference of the RGM candidate is observed in these gels, this is probably due to the presence of two other proteins in the selected spot. B: Deduced RGM peptide sequences

Figure 3: Nucleotide and amino acid sequence of RGM.

- A. Nucleotide sequence of RGM.
- B. Amino acid sequence of RGM. Peptides derived from microsequencing are highlighted in bold and peptides used for making polyclonal antibodies are underlined. Potential N-glycosylation sites and an RGD tripeptide, potential cell attachment site are underlined by asterics.
- C. Schematic view of the RGM protein. Hydrophobic domains are present at the N- and C- termini of the protein. Epitopes of the two polyclonal anti-RGM antibodies are demarcated.

Figure 4: The polyclonal and the monoclonal RGM antibody recognize the same 33 kDa protein.

- A. The anti-RGM1 antibody binds to a GPI-anchored CRD- (cross reacting determinant) positive 33 kDa protein. Left blot: An anti-CRD antibody binds to a low abundant, 33 kDa protein (arrow), present in the E (PI-PLC supernatant) but not the C fraction (control supernatant). Right blot: Anti-RGM1 staining of a GPI-anchored 33 kDa protein on a western blot with supernatant from E9/E10 chick brain membranes.

B. The GPI-anchored 33 kDa antigen of the anti-RGM1 antibody is more abundant in posterior (pos.) than in anterior (ant.) tectal membranes. Left blot: rabbit preimmune serum did not bind to any protein on western blots with PI-PLC supernatant protein from anterior and posterior tectum. Right blot: Anti-RGM1 binding to a 33 kDa protein. E = PI-PLC supernatant from tectal membranes, C = control supernatants from tectal membranes.

C. Anti-RGM1 and F3D4 recognize the same antigens in tectal membranes. Left blot: F3D4 staining of tectal membrane proteins. A double band at 33 kDa (lower arrow) and a hardly visible band at 35 kDa (upper arrow) are recognized.

Right blot: Anti-RGM1 staining reveals the same staining pattern with 33 and 35 kDa antigens (arrows). Contrary to the membrane fraction, where 3 different protein bands are observed, only one band is detected in most western blots with PI-PLC supernatants.

For detection on western blots, a secondary, alkaline phosphatase - conjugated antibody was used and NBT (nitro blue tetrazolium) and BCIP (bromochloroindolyl phosphat) was used for the colour reaction.

Figure 5: The RGM anti-sense probe hybridizes to an mRNA with graded expression along the antero - posterior axis.

A, B: RGM-mRNA is expressed in a periventricular gradient in the tectum of an E9 chick embryo. In a more superficial layer (arrows), RGM is also expressed but at much lower level. The anterior tectal pole is to the right, the posterior to the left.
C,D: No staining is detected with the RGM sense probe, on parallel cryostat sections from E9 chick tecta. The anterior tectal pole is to the right, the posterior to the left.

Figure 6: Recombinant RGM induces collapse of retinal growth cones.

A: RGC axons were grown on laminin-coated coverslips and affinity purified recombinant RGM was added at a final concentration of 10ng/ml. More than 90 % of temporal retinal growth cones are collapsed.

B: Neighboring, RGM - free fractions from affinity purification did not induce collapse of temporal growth cones. Supernatants from cos-7 cells transfected with an empty plasmid, did not possess any collapse-inducing activity (data not shown).

In A and B, retinal axons and growth cones were stained with the F-actin stain Alexa-Phalloidin.

Figure 7: Recombinant RGM guides temporal retinal axons in the stripe assay.

A, B: Temporal retinal axons avoid the RGM-containing stripes (demarcated with red fluorescent beads). Membranes from RGM - transfected cos-7 cells (marked with beads) and anterior tectal membranes were used to prepare striped carpets.

C, D: Temporal retinal axons do not show any avoidance reaction, when membranes from cos-7 cells, transfected with an empty plasmid (red beads) were used.

In A - D, striped membrane carpets were in addition coated with laminin to enhance retinal axon growth in accordance with a previous protocol (Monschau et al. 1997).

Fig. 8: RGM staining in endothel of (human) brain.

RGM immunoreactivity was detected in endothelial and vascular smooth muscle cells (SMC), both, in healthy, neuropathological unaltered control brains and injured brains, suggesting a constitutive, physiological role in vascular homeostasis.

Fig. 9: RGM expression in a lesion of a human being deceased due to severe brain injury (1-2 hours after his death). RGM expression on infiltrating cells from the immune system.

Upregulation of cellular RGM expression correlated with the time course and appearance of infiltrating leukocytes and activation of microglia/macrophages after injury (Stoll et al., 1998).

Early after injury (up to 2.5 days), RGM immunoreactivity was found on leukocytes of granulocytic, monocytic and lymphocytic origin in vessels within ischemic tissue.

Paralleled by edema formation, up to 1-7 days, RGM-positive cells were found extravasating outside the vascular walls into the focal ischemic lesioned parenchyma. In perivascular regions, RGM-positive cells formed clusters in the Virchow-Robin spaces from day 1-7, which subsided later. These peri-vascular cells, also referred to as adventitial or perithelial cells are characteristically alert immune cells (Kato and Walz, 2000; Streit et al., 1999).

Fig. 10: RGM expression in a brain lesion (human).

With increasing time after brain injury, most remarkable changes corresponded to areas of ongoing scar formation. In these areas, well defined extracellular RGM-positive laminae and RGM-positive fibroblastoid and reactive astrocytic cells were visible condensing adjacent to the border zone. These RGM-positive laminae increased in magnitude and regional extend over time.

The Examples illustrate the invention.

Example I: Microsequencing of an RGM candidate

To separate RGM from the A-ephrins, a combination of two different ionexchange columns was employed. RGM, in contrast to the A-ephrins, bound to a strong cation exchanger and was eluted at a salt concentration of 200 - 400 mM NaCl. After incorporation of RGM into lecithin vesicles, strong collapse-inducing activity was observed in RGM-fractions (fractions 4 + 5, Fig. 1) but not in neighboring RGM-free fractions (fraction 6, Fig. 1). Neither ephrin-A5 nor ephrin-A2 was present in these fractions, proving thereby that RGM function does not require presence of the A-ephrins.

To get peptide sequences from RGM, microsequencing of all proteins, (cleaved from the membrane by treatment with the enzyme PI-PLC and having a molecular weight of 30 – 35 kDa and an isoelectric point between 7 and 9 was carried out). To this aim, anterior and posterior membranes from embryonic chick tecta (E9/10) were prepared with some modifications as described previously (Walter, Development 101, (1987),

685-96) and membrane pellets were subject to treatment with enzyme PI-PLC (E fraction) or buffer alone (C fraction). In particular, Membranes from embryonic chick tecta (E9/10) were prepared with some modifications as described previously (Walter et al., 1987). All steps were performed at 4°C. Tecta from 100 chick embryos were isolated and were divided into three parts equal in length along the anterior-posterior axis. The middle tectal parts were discarded and the anterior and posterior parts were worked up separately. Membranes were washed with PBS containing protease inhibitors and were centrifuged. Tectal membrane pellets were resuspended in triethanolamin buffer and were treated with the enzyme PI-PLC (50 mU Boehringer Mannheim/Roche Diagnostics GmbH), to remove glycosylphosphatidylinositol-anchored (GPI-anchored) proteins from the membranes. No PI-PLC was added to the other anterior and posterior tectal membrane fractions, the control-fractions (C). Enzyme (E) and control (C) fractions were incubated at 37°C for 1.5 hours and membrane suspensions were centrifuged at 400.000 xg in a Beckmann TLA 100.3 rotor. Supernatants were collected and their protein concentrations were determined (Bradford 1976, modified by Zor and Selinger, 1996). Supernatants were precipitated with ice cold 10% trichloroacetic acid, were centrifuged and protein pellets were washed in ethanol-ether (1:1 v/v) and solubilized in sample buffer (8.5 M urea, 5% β-mercaptoethanol, 2.5 % ampholytes pH 3-10, 2% NP 40).

E fractions and C fractions were loaded onto two dimensional gels, and after silver staining candidate proteins in the E-fractions (Fig. 2A, arrows) were cut out and subject to in gel tryptic digestion and nanoelectrospray ionization (Wilm, Nature 379 (1996), 466-9).

In detail, said 2D gelectrophoresis and the protein sequence analysis was carried out as outlined herein below:

Tectal proteins resuspended in sample buffer, were separated using two dimensional gel electrophoresis. 20 µg of tectal protein was loaded on each gel. Non-equilibrium pH gradient electrophoresis (NEPHGE) followed by SDS-PAGE in the second dimension was performed as described by Boxberg (1988). After the SDS PAGE, gels were stained by a modified silver staining protocol from Heukeshoven and Dernick (Heukeshoven & Dernick, Electrophoresis 9, (1988), 372-375).

Silver-stained proteins in the 2D gels, with a basic isoelectric point and a molecular weight of 33/35 kDa, present in the PI-PLC treated E fraction but not in the C fraction, were cut out using a sharp and sterile scalpel.

Microsequencing was done using the technique of nano-electrospray tandem mass spectrometry as previously described (Wilm et al., 1996). The protein spots were digested in gel by trypsin and the resulting peptides were adsorbed and stepwise eluted into the electrospray source for mass spectral analysis. Nanoelectrospray was performed on an API III (Perkin-Elmer) mass spectrometer as described by Wilm and Mann (Wilm & Mann, Anal. Chem. 68 (1996), 1-8). After selecting an ionized peptide from the peptide mixture, the peptide was fragmented and the peptide fragments were analysed.

Mass spectrometric microsequencing of ionized peptides from the spot marked by arrows in figure 2, yielded ten different peptides, with lengths of 5 - 14 amino acids as shown in Fig. 2B; (SEQ ID NOs 1-10). The selected spot, was present in anterior and posterior PI-PLC supernatantes at similar levels. RGM is however more abundant in posterior than in anterior tectal membranes and the disappearance of the ap-difference in the 2D - gels was most likely caused by two different proteins unrelated to RGM and present in the selected spot.

Example II: Cloning of the RGM Gene

Three out of the ten peptide sequences (SEQ ID NOs 1 to 10) obtained by nanoelectrospray tandem mass spectrometry were used for synthesis of degenerate oligonucleotide primers and PCR experiments were performed as follows: Three out of the ten peptide sequences obtained by nanoelectrospray tandem mass spectrometry were used for synthesis of degenerate oligonucleotide primers and their complementary sequences.

P1F: 5'-ATGCC(AGCT)GA(AG)GA(AG)GT(AGCT)GT(AGCT)-3' (SEQ ID NO:11)

P1R: 5'-TT(AGCT)AC(AGCT)AC(CT)TC(CT)TC(AGCT)GGCAT-3' (SEQ ID NO:12)

P2F: 5'-GA(CT)AC(AGCT)TT(CT)CA(AG)AC(AGCT)TG(CT)AA-3' (SEQ ID NO:13)

P2R: 5'-TT(AG)CA(AGCT)GT(CT)TG(AG)AA(AGCT)GT(AG)TC-3' (SEQ ID NO:14)

P3F: 5'-AA(CT)CA(AG)CA(AG)(CT)T(AGCT)GA(CT)TT(CT)CA-3' (SEQ ID NO:15)
P3R: 5'-TG(AG)AA(AG)TC(AGCT)A(AG)(CT)TG(CT)TG(AG)TT-3' (SEQ ID NO:16)

Moloney murine leukemia virus reverse transcriptase and random hexamer primers were used to synthesize single-stranded cDNA from E9 chick tectum total RNA. Combinations of forward (F) and reverse (R) primers were added to the cDNA and PCR amplification was done using Taq polymerase. The following PCR conditions were used: an initial denaturation step at 95°C for 5 min followed by 30 cycles of 95°C for 40 s, 50°C for 1 min, 72°C for 2 min. The PCR products were cloned into the pGEM T vector (Promega) and four positive clones were sequenced using the ALF express sequencer (Pharmacia). The sequence yielded an ORF, containing most of the peptide sequences obtained by microsequencing. The 459 bp fragment was used for screening a cDNA library to obtain the full length sequence and for further analysis such as Northern blotting and in situ hybridization.

The PCR products were loaded onto agarose gels stained with ethidium bromide and a PCR product of 459 bp in length, was obtained and cloned into the pGEM T vector. After sequencing, most of the peptide sequences were found in the PCR product, confirming that the correct candidate was amplified. The 459 bp fragment was used for screening an E14 chicken brain cDNA library. Positive clones contained an insert of approximately 4 kb and sequencing confirmed the presence of the 459 bp fragment and additional downstream sequences, including a stop codon. Upstream sequences were obtained by performing 5'-RACE.

In detail, the 459 bp probe was used to screen 500.000 plaques of an E14 chicken brain library, cloned in the λ Zap vector. After two screening rounds, eight single plaques were isolated and the related inserts were cloned into the Bluescript vector using the rapid excision kit (Stratagene). The positive clones, analysed by restriction digestions, contained an insert of approximately 4 kb and sequencing confirmed the presence of the 459 bp fragment and additional downstream sequences, including a stop codon. To get the sequence of the region upstream of the 459 bp fragment, a 5'-RACE was performed according to the manufacturer's protocol using the RACE kit from Boehringer Mannheim and total RNA from E9 chick tecta. A 700 bp band was amplified, purified, cloned into pGEM T vector,

and 5 positive clones were sequenced. The sequence had an ORF with two methionines which could act as potential start sites. The full length sequence of RGM was confirmed independently several times.

For *in situ* and Northern blot experiments, the 459 bp fragment was cloned into the Bluescript KS vector (Stratagene) and anti-sense and sense probes were produced by using the SP6 and T7 polymerases, respectively.

This 5'-RACE yielded an ORF with two methionines, potential start sites. The complete ORF of RGM is 1302 nucleotides in length and encodes a protein consisting of 434 amino acids (Fig.3A; SEQ ID NO:17). Two hydrophobic domains are present at the N-terminus and C-terminus, respectively (Fig. 3B; SEQ ID NO:18), and two different algorithms suggested that the N-terminal hydrophobic domain encodes a signal peptide (best cleavage site predicted: at aa 29), the C-terminal domain, a GPI-anchor domain (best cleavage site predicted: at aa 406). RGM has no significant homology to any other protein, present in the databases and does not carry any specific domain or motif, except an triamino acid motif, the RGD site, a potential cell attachment site (Ruoshlahti, Annu. Rev. Cell Dev. Biol. 12 (1996), 697-715). Preliminary results suggest that this site is dispensable for RGM function. Polyclonal antibodies, named anti-RGM1 (against aas: 276 - 293) and anti-RGM2 (against aas: 110 - 130), raised against two peptides of the recombinant RGM molecule, recognize a GPI-anchored 33 kDa molecule, which is present at higher levels in posterior than in anterior tectal membrane PI-PLC supernatants (Fig. 4A). In membrane fractions at least three protein bands appear, a double band at 33 kDa and a single band at 35 kDa. These protein bands are recognized by the polyclonal anti-RGM1 antibody and the monoclonal F3D4 antibody (Müller (1996), loc. cit) (Fig. 4B). Both antibodies show identical staining patterns on western blots and immunoprecipitation experiments with anti-RGM1 resulted in pull down of a GPI-anchored, F3D4-positive protein. These results prove, that the antigens of the F3D4 monoclonal antibody and of the anti-RGM1 polyclonal antibody are identical.

RGM is the first member of a new class of axon guidance molecules, sharing no sequence homology with ephrins, netrins, slits, semaphorins and any other axon guidance molecules.

The corresponding human RGM sequence (SEQ ID NO:20) could be deduced by screening the human genome database with the deduced chicken RGM sequence.

Example III: RGM mRNA is expressed in a gradient in the optic tectum

To analyse expression of RGM-mRNA in the tectum opticum, an RGM anti-sense probe was used in *in situ* hybridization experiments on cryostat sections from E9 chick tecta. Strongest staining is observed in the periventricular layer, surrounding the tectal ventricle and staining intensity is much stronger in posterior tectum than in anterior tectum (Fig. 5A, B). Cell bodies of radial glial cells are located in the periventricular layer and the staining pattern confirms previous data using the monoclonal F3D4 antibody, where staining of glial endfeet and of glial cell bodies was observed (Mueller; (1996), loc. cit.; Mueller, (1997), loc. cit.). In a more superficial layer, a much weaker staining is detected with the RGM anti-sense probe but a differential expression between anterior and posterior tectal poles is hard to detect in this layer. In this layer tectal neurons are RGM-positive. This is in line with the expression of RGM by a subpopulation of tectal neurons. Overall, the staining pattern with the RGM anti-sense probe looks very similar to the expression pattern of ephrin-A5 with both messages being found in a periventricular and in a more superficial tectal layer. No staining is detectable with the RGM-sense probe.

On northern blots with tectal RNA, the RGM anti-sense probe marked two transcripts at 5.5 and 6.1 kb. Both messages are down-regulated at E14 with the smaller message being no longer detectable and the larger transcript being clearly present, albeit at lower levels.

RGM is active in *in vitro* assays and shows a graded expression in the tectum opticum of vertebrates. Based on Southern blot data it is assumed that there are least two additional family members which might have similar guidance activity. (see Fig. 11)

Example IV: Recombinant RGM is Active in Collapse and Stripe Assay

To analyse the function of recombinant RGM, the full length RGM cDNA was used to transfect cos-7 cells with a lipofection procedure. The full length RGM cDNA was cloned into the KpnI site of the expression vector pTriEx-1 (Novagen). Cos-7 cells were transfected with the pTriEx-1 plasmid containing RGM cDNA or with the empty plasmid using the Superfect transfection reagent (Qiagen) according to the manufacturer's protocol. The DNA-Superfect mixture was added to Cos-7 cells growing in 10 cm dishes. 2 hours later medium was removed, cells were washed with PBS and grown for an additional 48 hours in fresh medium. Conditioned medium was collected, run over an RGM-affinity column and RGM-containing fractions and RGM-free control fractions were directly used in collapse assay experiments. For stripe assay experiments, RGM-transfected Cos-7 cells and empty plasmid transfected cells were washed with PBS and harvested using a rubber policeman in the presence of homogenization buffer containing protease inhibitors. Conditioned medium of cos-7 cells transfected with the RGM-pTriEx-1 plasmid was collected and run over an anti-RGM1 antibody column. Eluted fractions were evaluated with a sensitive and rapid dot blot assay and RGM-positive fractions were added to retinal axons growing on a laminin substratum. At a final concentration of 10 ng/ml, soluble RGM induced collapse of 90% of temporal RGC growth cones (Fig. 6A). Neighboring, RGM-free fractions or conditioned and concentrated supernatants from cos-7 cells transfected with the empty plasmid did not possess any collapse-inducing activity (Fig. 6B). Recombinant RGM is active in soluble form, is a strong difference between RGM and the A-ephrins and suggests a role for a chemotropic mechanism, in establishing the retinotectal map.

For preparation of striped membrane carpets, membranes from RGM- or mock-transfected cells were used. Carpets consisting of alternate lanes of membranes from mock-transfected cos-7 cells and from RGM-transfected cells were offered to temporal and nasal RGC axons. To enhance the poor outgrowth-stimulating activity of cos-7 membranes, anterior tectal membranes or laminin were added. Collapse assay and stripe assays were prepared and employed as follows: The collapse assay was performed as described (Cox, (1990), loc. cit.; Wahl, J. Cell Biol. 149(2) (2000), 263-70). 5 µl of the RGM-positive fraction from the RGM-cos supernatant or supernatant from control cos cells or RGM-free fractions, was

added to the retinal cultures. One hour later cultures were fixed by carefully adding 1 ml of fixative (4% paraformaldehyde, 0.33 M sucrose, pH 7.4). 4 – 12 hours later, cultures were washed and stained by Alexa-Phalloidin (Molecular Probes), following the recommendations of the manufacturer. Stained cultures were stored on a computer using a CCD camera and the images were analysed with the SIS analysis imaging software.

Stripe assay experiments were performed as previously described by Walter et al. (1987). Membrane carpets consisted of lanes of anterior tectal membranes mixed with membranes from RGM-transfected cos cells (ratio: 1:1), alternating with lanes consisting of anterior membranes mixed with membranes from empty plasmid transfected cos cells (ratio 1:1). In an alternative protocol, membrane carpets consisting of alternating lanes of membranes from RGM-transfected cos cells and of control cos membranes, were incubated for 2 hours at 37°C with 20 µg/ml laminin (Becton-Dickinson). Before use, the carpets were washed with Hank's buffer (2x).

On these carpets, temporal RGC axons, but not nasal axons, showed a clear repulsive avoidance behaviour, growing on the RGM-free membrane stripes (Fig. 7 A - D). These results demonstrate, that the recombinant RGM protein is not only active in collapse but also in stripe assays.

RGM shares with the A-ephrins A2 and A5 the GPI-anchor, the graded expression and functional activity in two different *in vitro* systems. Its activity is however different from the two A-ephrins in other respects. The specificity of its activity is not restricted to temporal axons and growth cones. Nasal axons and growth cones also react, albeit at higher RGM concentrations. This is in line with the previous observations, that temporal retinal axons react more strongly to RGM than nasal retinal axons (Stahl, (1990), loc.cit). For ephrin-A5, a slight difference in sensitivity of temporal and nasal retinal axons has been observed, this difference is however not as pronounced as with RGM (Drescher, Cell 82 (1995), 359-70). Besides the stronger concentration dependancy of RGM function, another crucial difference is that RGM, in contrast to both ephrin-A5 and ephrin-A2, seems to be active in soluble form and apparently does not require aggregation to stimulate its currently unknown retinal receptor. These *in vitro* results underscore the difference between ephrins and RGM. In the stripe assay, inactivation of RGM using the F3D4

monoclonal antibody and the chromophore-assisted laser inactivation (CALI) method, resulted in complete neutralization of repulsive guidance activities of posterior tectal membranes in more than 50% of the experiments (Mueller, (1996), loc. cit.) F3D4 however neither binds ephrin-A2 nor ephrin-A5 (Mueller, (1997), loc. cit.) and it was therefore suggested that the A-ephrins and RGM somehow interact in special membrane domains to which they are recruited by their GPI-anchors. Such a colocalization could explain the result, that inactivation of RGM lead in addition to inactivation of ephrin-A2 and ephrin-A5 and could explain the complete neutralization observed in the stripe assay experiments (Mueller, (1996), loc. cit.). The functional relationship of RGM with ephrin-A2 and ephrin-A5 and the in vivo role of RGM need to be addressed, especially since both ephrins have been shown to be important molecular determinants for topographic map formation in vertebrates (Nakamoto, Cell 86 (1996), 755-66; Frisen, Neuron 20 (1998), 235-43; Feldheim, Neuron 21 (1998), 563-74; Picker, Development 126 (1999), 2967-78; Feldheim, Neuron 25 (2000), 563-74; Brown, Cell 102 (2000), 77-88). There are however evidences from two vertebrates, which suggest that other factors, besides the ephrins, are required for formation of the retinotectal map. Deletion of either the ephrin-A2 or the ephrin-A5 gene in mice, resulted in mapping phenotypes with some retinal axons forming ectopic termination zones in the superior colliculus (SC), the mammalian homologue of the optic tectum, and with nasal retinal axons overshooting the SC and terminating in the inferior colliculus. In ephrin-A2^{-/-} mice, temporal axons showed mapping errors with ectopic termination zones, but nasal axons did not show any mapping errors in contrast to the ephrin-A5^{-/-} mice which had defects in topographic mapping of nasal but not temporal axons (Frisen, (1998), loc.cit.; Feldheim, (2000), loc. cit.). Deletion of both genes should therefore result in a much more disturbed mapping of both nasal and temporal retinal axons along the anterior-posterior axis of the SC . This is actually observed in double mutant ephrin-A2^{-/-} A5^{-/-} homozygotes but a topographic bias of both nasal and temporal axons was still present, with the majority of temporal and nasal retinal axons being confined to their anterior and posterior tectal halves, respectively (Feldheim, (2000), loc. cit.; Goodhill, Neuron 25 (2000), 501-3). These results point to a role of RGM as one of the additional

factors required for mapping along the anterior-posterior axis. Such a role is supported by the graded anterior-low posterior-high expression of this molecule in the SC of mammals (Mueller, (1997), loc. cit.).

The zebrafish mutant acerebella (ace) is mutant in fgf8 and lacks the midbrain-hindbrain boundary region and the cerebellum (Reifers, Development 125 (1998), 2381-95; Picker, (1999), loc. cit.). As a result the tectum is much smaller in ace mutants than in wildtype and the expression levels of all three zebrafish A-ephrins are changed with ephrin-A2 and ephrin-A5a being still expressed at low and anterior levels in ace tecta and with ephrin-A5b being completely eliminated (Picker et al., 1999). In ace mutant tecta, mapping of retinal axons along the anterior-posterior axis is normal in dorsal tectum and is not completely lost in ventral tectum, suggesting the involvement of other graded guidance cues, not seriously affected by the fgf8 mutation in the ace zebrafish mutants (Picker et al., 1999). Dorsoventral patterning in both zebrafish ace mutants and ephrin-A2^{-/-} A5^{-/-} double knock out mice is affected.

RGM, with its graded expression along the anterior-posterior axis of the tectum and its ability to function in a secreted and membrane-coupled way, is an important player for topographic map formation.

Example V:

Materials and methods

1. Patients

21 brains of patients with clinical history and neuropathologically confirmed diagnosis of focal cerebral infarctions and 25 brains of patients with traumatic brain injury were included in this study. Infarctioned brain tissue was derived from an updated stroke and trauma brain-bank (Table 1,2) reported previously (Postler et al., 1997, Beschorner et al., 2000). Tissue specimen procurement was performed according to the ethical guidelines of the University of Tuebingen. Patients with altered immune status because of immunosuppressive therapy or

meningitis/encephalitis were excluded from this study. As controls, the results were compared to tissue from corresponding areas of 4 normal non-ischemic brains described previously (Schwab et al., 2000). In addition to patient data, haematoxyline-eosine (HE), luxol fast blue (LFB) and iron (Fe) staining was used for evaluation of the typical histological features defined as standard indication of infarct (Kalimo et al., 1996) and trauma age (Graham and Gennarelli, 1996).

2 *Immunohistochemistry*

After formaldehyde fixation and paraffin-embedding, rehydrated 2 µm sections were boiled (in an 600W microwave oven) seven times for 5 min in citrate buffer (2,1g sodium citrate/liter, pH 7,4). Endogenous peroxidase was inhibited with 1% H₂O₂ in methanol (1:10; 15 min). Sections were incubated with 10% normal porcine serum (Biochrom, Berlin, FRG) to block non-specific binding of immunoglobulins. Monospecific polyclonal antibodies directed against RGM were diluted (1:10) in 1% BSA (bovine serum albumin) TBS (Tris-balanced salt solution, containing 0.025 M Tris, 0.15 M NaCl) and incubated over night at room temperature. Specific binding of the antibodies were detected with a secondary biotinylated swine anti-rabbit IgG F(ab)₂ antibody fragment 1:400 for 30 min (DAKO, Hamburg, FRG), followed by incubation with a peroxidase conjugated streptavidin-biotin complex (DAKO, Hamburg, FRG). The enzyme was visualized with diaminobenzidine as a chromogen (Fluka, Neu-Ulm, FRG). Sections were counterstained with Mayer's Hemalaun. Negative controls consisted of sections incubated in the absence of the primary antibody. Specificity of polyclonal RGM antibody was confirmed by inhibition of staining using human ischemic brain tissue after pre-incubation for 3h on ice with access of the cognate RGM peptide.

3. *Double Labeling Experiments*

In double labelling experiments, a cell-type or activation specific antigen was first labelled using the ABC procedure in combination with alkaline phosphatase conjugates. Specific antigens were labelled with antibodies against GFAP (glial

fibrillary acidic protein, monoclonal, Boehringer Mannheim, Germany, 1:100) to detect astrocytes, MBP (myelin basic protein, polyclonal, oligodendrocytes, Dako, 1:500) and CD68 (Dako, 1:100) for microglia/macrophage identification. Activated microglia/macrophages were detected with antibodies directed against HLA-DR, -DP, -DQ (MHC class II, DAKO, Glostrup, Denmark, 1:100) or MRP-8 (8-5C2, BMA, Augst, Switzerland, 1:100) (Postler et al., 1997). Lymphocytic subpopulations were classified with monoclonal antibodies against CD4 (T-helper lymphocytes, 1:10, Dako) and CD8 (T cytotoxic/suppressor lymphocytes, 1:500, Dako) and CD20 (pan B cell marker, 1:200, Dako). In order to detect extracellular basal lamina structures in vessels and during scar formation mouse laminin (1:500, Chemicon) antibodies were used and rabbit fibronectin (1:100, Dako) antibodies were used to detect matrix deposition. Furthermore, in order to characterize the cellular proliferation response, sections were incubated with the S phase specific PCNA (proliferating cell nuclear antigen, 1:100, Dako) monoclonal antibodies. Briefly, slices were deparaffinized, irradiated in a microwave oven for antigen retrieval and incubated with non specific porcine serum as described above. Visualization was achieved by adding biotinylated secondary antibodies (1:400) for 30 min and alkaline phosphatase conjugated ABC complex diluted 1:400 in TBS-BSA for 30 min. Consecutively, slices were developed with Fast-Blue BB salt chromogen-substrate solution yielding a blue reaction product. Between double labelling experiments, slices were irradiated in a microwave for 5 min in citrate buffer. Then RGM was immunodetected as described above.

4. *Evaluation and statistical analysis*

Data were calculated as means of labelled cells (MLC, \pm SEM) from border zones or remote areas of the same tissue section and were compared to normal control brains using the two-tailed unpaired student's t-test. Border zones were defined as peri-lesional areas adjacent to the developing necrotic core demarcating the region of major damage. RGM⁺ cells were counted in ten high power fields (HPF, $\times 200$ magnification with an eye-piece-grid representing 0.25 mm²).

Results

21 brains of patients with focal cerebral infarctions (FCI), 25 brains with traumatic brain injury (TBI) and 4 control brains were evaluated for RGM protein expression by immunohistochemistry.

1. *Healthy, neuropathological unaltered control brains*

In control brains without neuropathologically alterations, RGM immunoreactivity was detected on white matter fibres, oligodendrocytes, the perikarya of some neurons and RGM⁺ cells were also detected in the choroid plexus (Fig. 8) and ependyma. Only single cells were detected in peri-vascular spaces. Further, some smooth muscle cells and few endothelial cells but no astrocytes were labelled.

2. *Focal cerebral ischemia (FCI)*

It was analysed whether number and distribution of RGM expressing cells is altered after cerebral infarctions. Results suggested, that RGM expression is lesion-associated. Cellular RGM expression was confined to neurons, few reactive astrocytes and invading leukocytes. With the ageing of the lesions, RGM-positive extracellular laminae components were found in the constituting scar.

RGM-positive cells accumulated in infarctioned white matter, hemorrhagic areas, infarction core and peri-infarctional areas, respectively. Using the student's t-test, a significantly ($P<0.0001$) higher number of RGM⁺ cells was detected in peri-infarctional areas (MLC=24, SEM=1.1) than in remote areas (MLC=2, SEM=0.2) or control tissue (MLC=6, SEM=0.8). The morphological described peri-infarctional areas were part of the physiologically defined penumbra. In these areas the number of RGM-positive cells accumulated already up to day 1 ($p<0.0001$, MLC=31.93, SEM=2.3) reached their maximum 1.5-2.5 days (MLC=34, SEM=3.2) after infarction and remained elevated up to several weeks and months of survival

(MLC=11, SEM=1.4). Early after ischemic damage (up to 2.5 days), RGM immunoreactivity was predominantly found on neurons and leukocytes of granulocytic, monocytic and lymphocytic origin in vessels within ischemic tissue. Paralleled by edema formation, up to 1-7 days, RGM-positive cells were found extravasating outside the vascular walls into the focal ischemic lesioned parenchyma. In perivascular regions, RGM-positive cells formed clusters in the Virchow-Robin spaces from day 1-7, which subsided later. These peri-vascular cells, also referred to as adventitial or perithelial cells are characteristically alert immune cells (Kato and Walz, 2000; Streit et al., 1999). With lesion aging, from day 3 onwards, lesional RGM expression by few reactive astrocytes, was observed. At later stages, arising 1 week after infarction, extracellular RGM deposits were detected constituting neo-laminae localized to areas of ongoing scar formation. These RGM-positive laminae increased in magnitude and regional extend over time. With tissue reorganisation of the lesion, also "foamy", lipid loaded RGM-positive phagocytic RGM-positive microglia/macrophages were observed.

Upregulation of cellular RGM expression correlated with the time course and appearance of infiltrating leukocytes and activation of microglia/macrophages after injury (Stoll et al., 1998). Whereas upregulation of extracellular RGM expression correlated with the time course and the appearance of the scar after injury. In few cases (< 5% of counterstained nuclei) some reactive astrocytes restricted to the demarcating lesion core also expressed RGM.

3. *Traumatic brain Injury*

In patients who died after TBI, in accordance to cerebral infarction (FCI) the immunohistological evaluation revealed early cellular membranous, cytoplasmatic and nuclear RGM expression by leukocytes, few reactive astrocytes and neurons with strong staining of their perikarya, dendrites and axons (Fig. 9). During the observed time post TBI, within the necrotic core and the bordering peri-necrotic parenchyma accumulation of RGM-positive cells ($p<0.0001$) was detected in border zones (MLC=22, SEM=0.7) compared to remote areas (MLC=1, SEM=0.1)

and normal brain controls (MLC=5.8, SEM=0.8). Following TBI, RGM-positive cell numbers arose already during the first 24 hours ($p<0.0001$) where RGM-positive cell numbers reached maximum levels (MLC=29, SEM=0.9) and decreased subsequently. With increasing time after TBI, most remarkable changes corresponded to areas of ongoing scar formation (Fig. 10). In these areas, well defined extracellular RGM-positive laminae were visible condensing adjacent to the border zone. RGM immunoreactivity was also detected in endothelial and vascular smooth muscle cells (SMC) but no significant differences were observed between injured and control brains.

References as mentioned in the example herein above:

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Example VI: Change of tumor growth behaviour in mice

Hybridoma cells secreting the RGM-specific F3D4 monoclonal antibody were injected into the peritoneum of mice, primed with mineral oil. Normally the hybridoma cells continue to divide in the peritoneum and the hybridoma cells secreted large amounts of antibody, resulting in formation of large ascites tumors. Mice receiving the F3D4-producing hybridoma cells did not develop ascites tumors in the peritoneal cavity, but developed solid, adherent tumors. The F3D4 monoclonal antibody resulted in a change of phenotype of the tumorigenic hybridoma cells from a less invasive, non-adherent state to an invasive adherent state. Masking of endogenous RGM by the

antibodies secreted from the hybridoma cells, enabled adhesion and invasion of these tumor cells and was responsible for this outcome.

Example VII: Detection of a functional RGM fragment

RGM was cloned into the pTriEx vector and the vector was cut inside the polylinker side and inside the RGM sequence using SacI in the first step. After ligation of both ends the RGM containing vector was cut in the second step with SstI inside the RGM sequence and in the polylinker with PmlI. After ligation of both ends, the vector with the shorter RGM-fragments was transfected into COS7 cells. Cell lysates of these COS cells were purified using an anti-RGM1 affinity column and RGM-containing fractions were used in collapse assay experiments. A fragment as described in SEQ ID NO:19 was active in said assays.

Example VIII: Detection of further RGMs

A publicly available computer database at the National Center for Biotechnology Information (NCBI, USA) was used to identify human genes homologous to chicken RGM, employing the information and data illustrated in the examples herein above. A search strategy based on the Blast algorithm (NCBI) resulted in three human genes located on chromosomes 1, 5 and 15. The corresponding contigs are NT_021932.5 (RGM3), NT_029283.2 (RGM2) and NT_010370.5 (RGM1), respectively. cDNA sequences for RGM 1, 2 and 3 were derived from these genomic sequences by omitting introns and fusing the remaining exons.

Corresponding amino acid and nucleotide sequences for human RGM2 are illustrated in appended SEQ ID NOs: 22 and 23. Human RGM3-sequences are shown in SEQ ID NOs: 24 and 25.

Claims

1. Use of a modulator of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue, associated with seizures associated with angiogenic disorders or disorders of the cardio-vascular system.
2. The use of claim 1 wherein said diseases or conditions associated with the degeneration or injury of vertebrate nervous tissue are selected from the group consisting of neurodegenerative diseases, nerve fiber injuries and disorders related to nerve fiber losses.
3. The use of claim 2, wherein said neurodegenerative disease is selected from the group consisting of motorneuronal diseases (MND), ALS, Alzheimers disease, Parkinsons disease, progressive bulbar palsy, progressive muscular atrophy, HIV-related dementia and spinal muscular atrophy(ies), Down's Syndrome, Huntington's Disease, Creutzfeldt-Jacob Disease, Gerstmann-Straeussler Syndrome, kuru, Scrapie, transmissible mink encephalopathy, other unknown prion diseases, multiple system atrophy, Riley-Day familial dysautonomia wherein said nerve fiber injuries are selected from the group consisting of spinal cord injury(ies), brain injuries related to raised intracranial pressure, trauma, secondary damage due to increased intracranial pressure, infection, infarction, exposure to toxic agents, malignancy and paraneoplastic syndromes and wherein said disorders related to nerve fiber losses are selected from the group consisting of paresis of nervus facialis, nervus medianus, nervus ulnaris, nervus axillaris, nervus thoracicus longus, nervus radialis and for of other peripheral nerves.

4. The use of claim 1, wherein said disease or condition associated with seizures is epilepsy.
5. The use of claim 1, wherein said disease or conditions associated with angiogenic disorders are selected from the group consisting of ischemic disorders, infarction, disorders leading to the progression of vascular plaque formation in cardio-vascular, cerebrovascular and/or nephro-vascular disorders or disorders wherein the permeability of the blood-brain barrier has to be regulated.
6. The use of claim 1, wherein said disorders of the cardio-vascular system comprises disorders of the blood-brain barrier, brain oedema, secondary brain damages due to increased intracranial pressure, infection, infarction, ischemia, hypoxia, hypoglycemia, exposure to toxic agents, malignancy, paraneoplastic syndromes.
7. Use of a modulator of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for remyelination.
8. Use of a modulator of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for modifying and/or altering the differentiation of neuronal stem cells and/or their progenitors.
9. The use of any one of claims 1 to 8, wherein said modulator is an antibody or a fragment or a derivative thereof, is an aptamer, is a specific receptor molecule capable of interacting with a polypeptide having or comprising the amino acid sequence of SEQ ID NO: 18, 20, 23 or 25 or with a functional fragment or

derivative thereof or is a specific nucleic acid molecule interacting with a polynucleotide encoding said polypeptide.

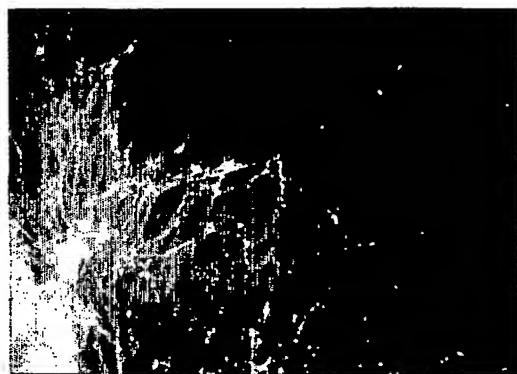
10. Use of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding such polypeptide or fragment as derivative for the preparation of a pharmaceutical composition for preventing or treating diseases or conditions associated with excessive collateral sprouting of nerve fibres.
11. Use of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing or treating tumor growth or formation of tumor metastases.
12. Use of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for preventing, alleviating or treating diseases or conditions associated with the activity of autoreactive immune cells or with overreactive inflammatory cells.
13. Use of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for the treatment of inflammation processes and/or allergies, for wound healing or for the suppression/alleviation of scar formation.
14. Use of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a

polynucleotide encoding said polypeptide or fragment or derivative as a marker of stem cells.

15. Use of a polypeptide having or comprising the amino acid sequence of SEQ ID NOs 18, 20, 23 or 25 or of a functional fragment or derivative thereof or of a polynucleotide encoding said polypeptide or fragment or derivative for the preparation of a pharmaceutical composition for alleviating, preventing and/or treating homoestatic and/or bleeding disorders and/or vascular damage.
16. The use of any one of claims 10 to 15, wherein said polypeptide or fragment or derivative is soluble.
17. Use of an antibody or a fragment or a derivative thereof, is an aptamer, is a specific receptor molecule capable of interacting with a polypeptide having or comprising the amino acid sequence of SEQ ID NO: 18, 20, 23 or 25 or with a functional fragment or derivative thereof, or is a specific nucleic acid molecule interacting with a polynucleotide encoding said polypeptide for the preparation of a diagnostic composition for detecting neurological, neurodegenerative disorders or dispositions thereto.

Fig.1a

RGM fractions induce growth cone collapse



Fraction 4

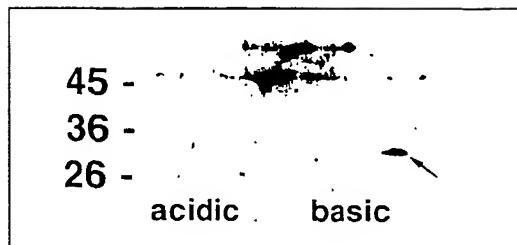


Fig.1b



Fraction 5

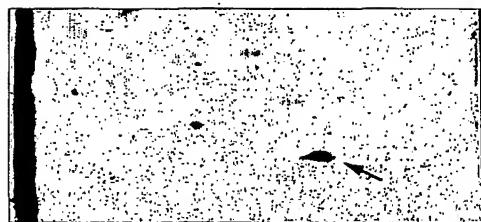


Fig.1c



Fraction 6

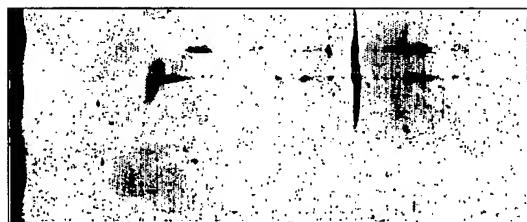
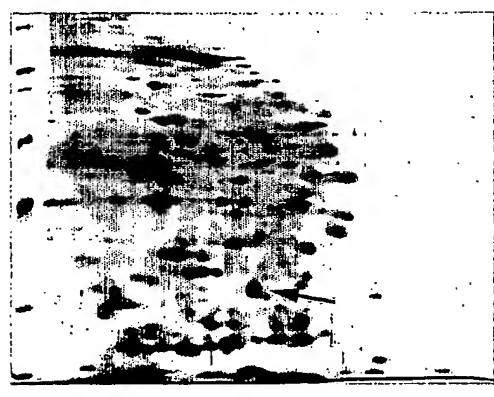
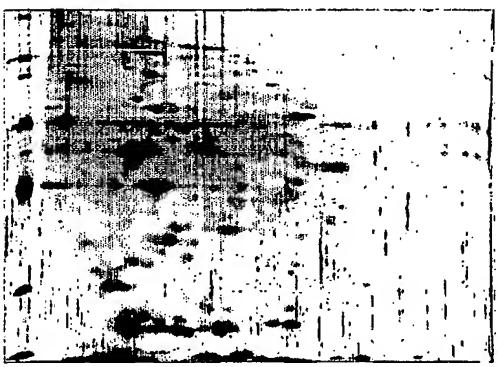
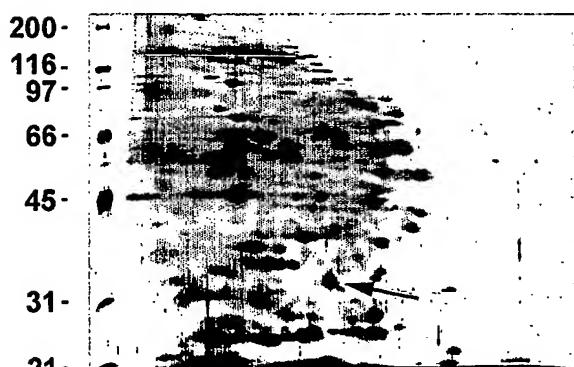


Fig.2a

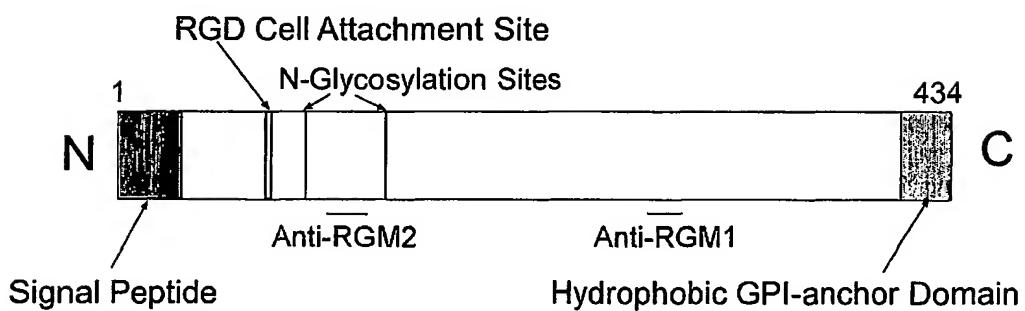
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IP 3 → 10

Fig.2b**RGM Peptide Sequences**

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TFTDTFQ	(SEQ ID NO: 2)
MPEEVVNAVEDR	(SEQ ID NO: 3)
LTLLFK	(SEQ ID NO: 4)
TFTDTFQTCK	(SEQ ID NO: 5)
(GC)PLNQQQLDFQTMR	(SEQ ID NO: 6)
AEMDE	(SEQ ID NO: 7)
PEAFTYE	(SEQ ID NO: 8)
HLEYR	(SEQ ID NO: 9)
QGLYL	(SEQ ID NO:10)

Fig.3

RGM = Repulsive Guidance Molecule

New Protein with no Sequence Homology
Many Cysteines (3.8%)
Many Prolines (5.5%)

Fig. 4

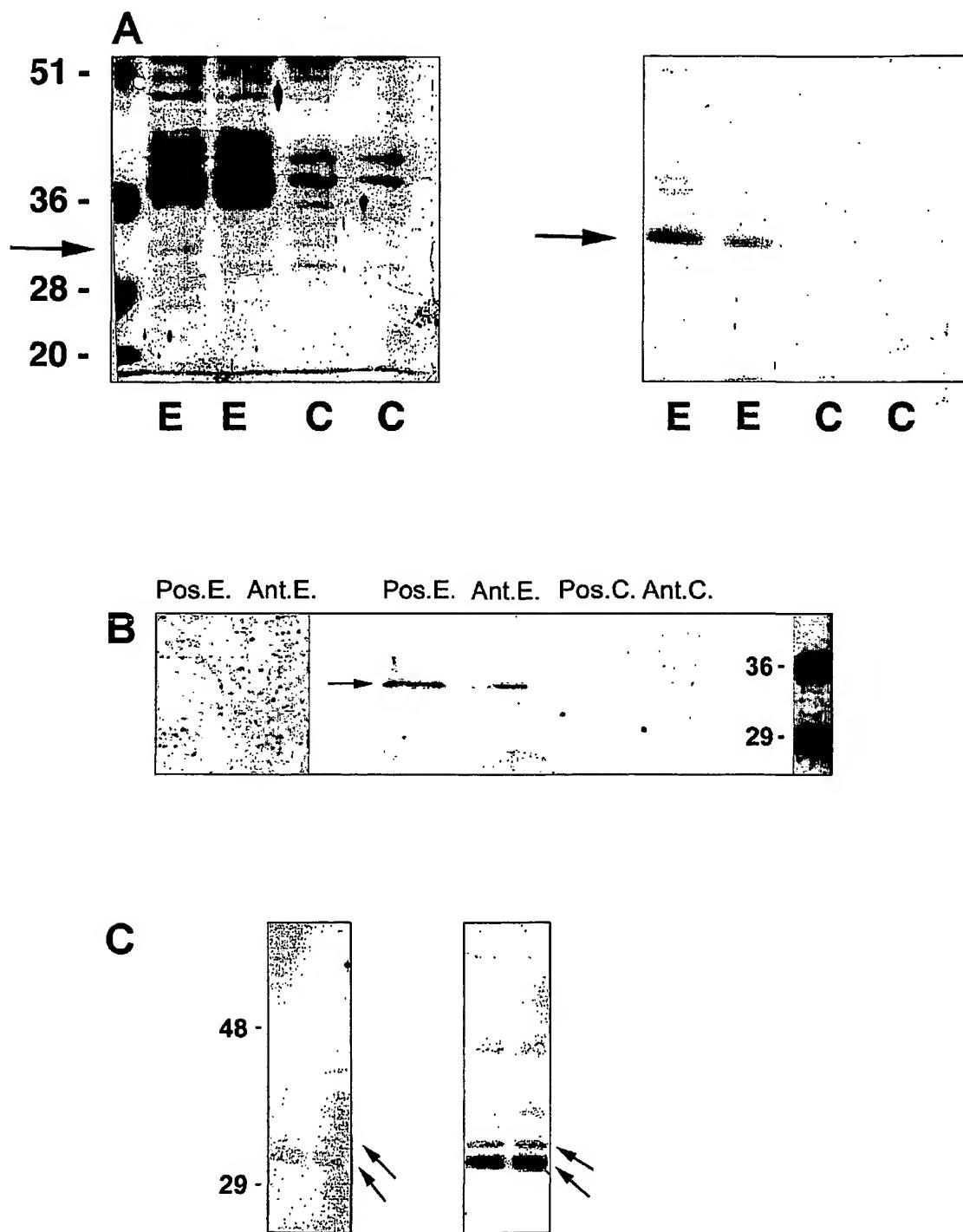


Fig.5

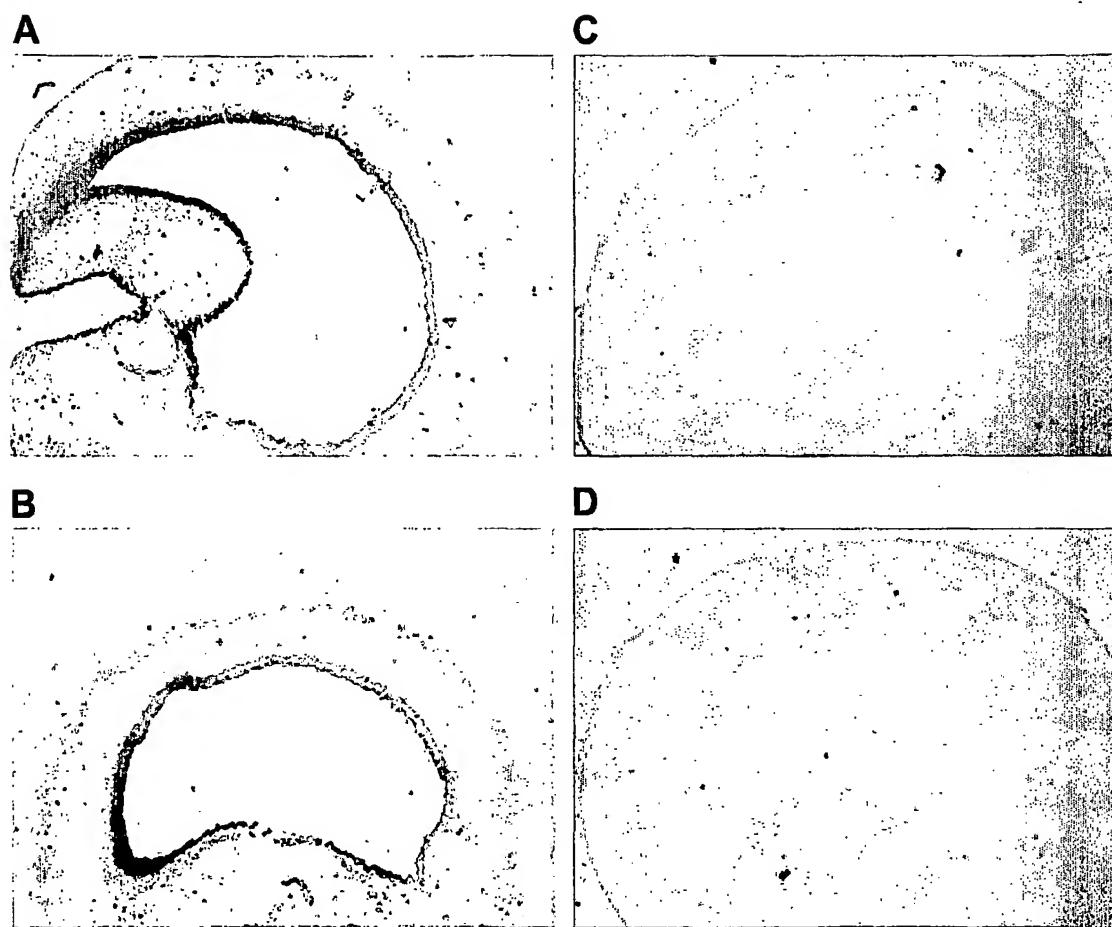


Fig.6

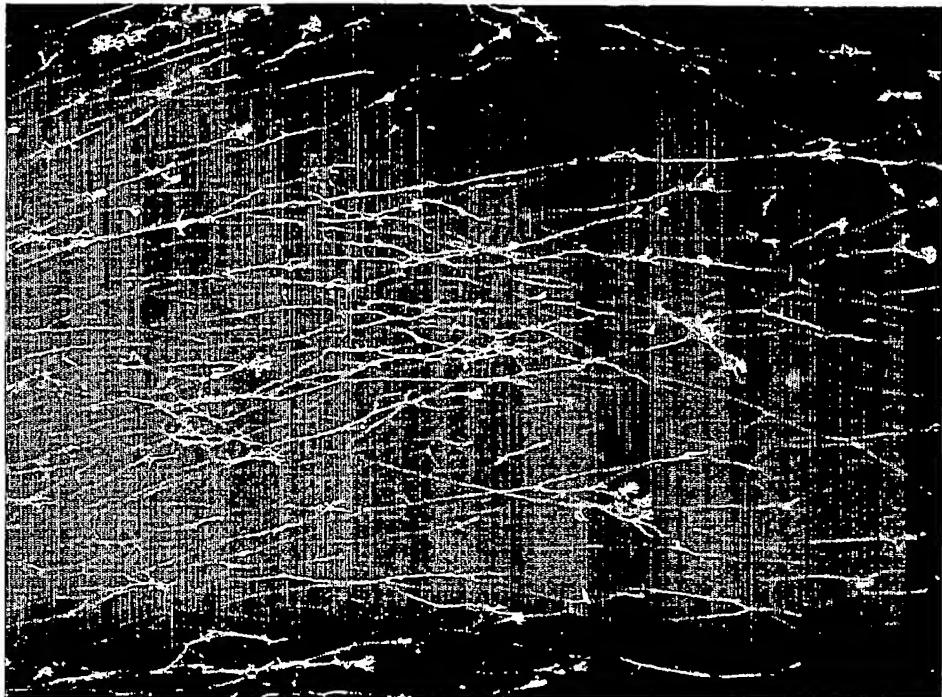
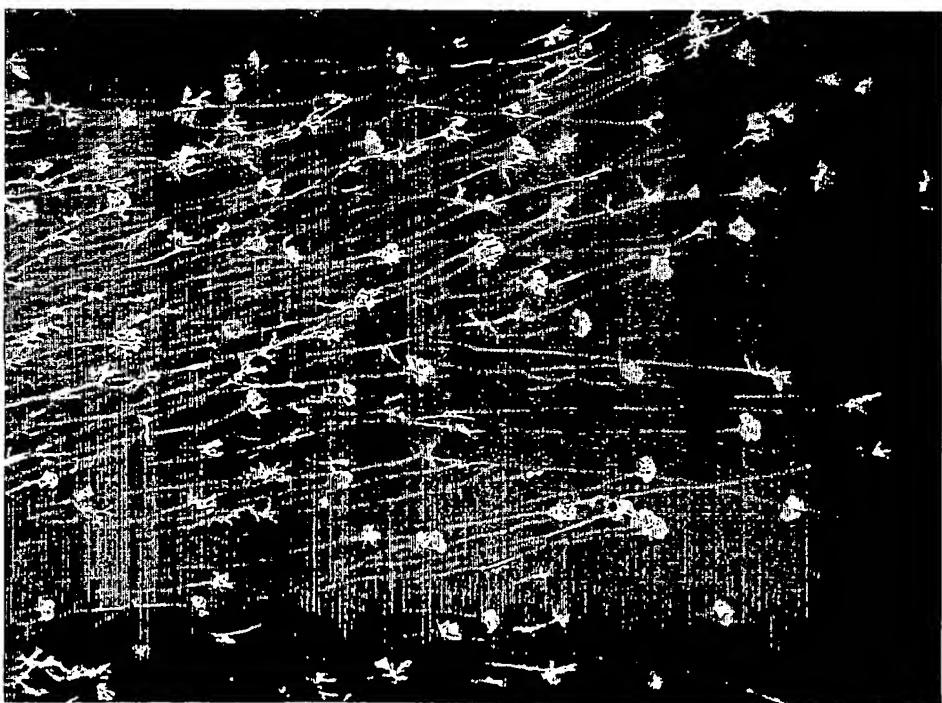
A**B**

Fig. 7

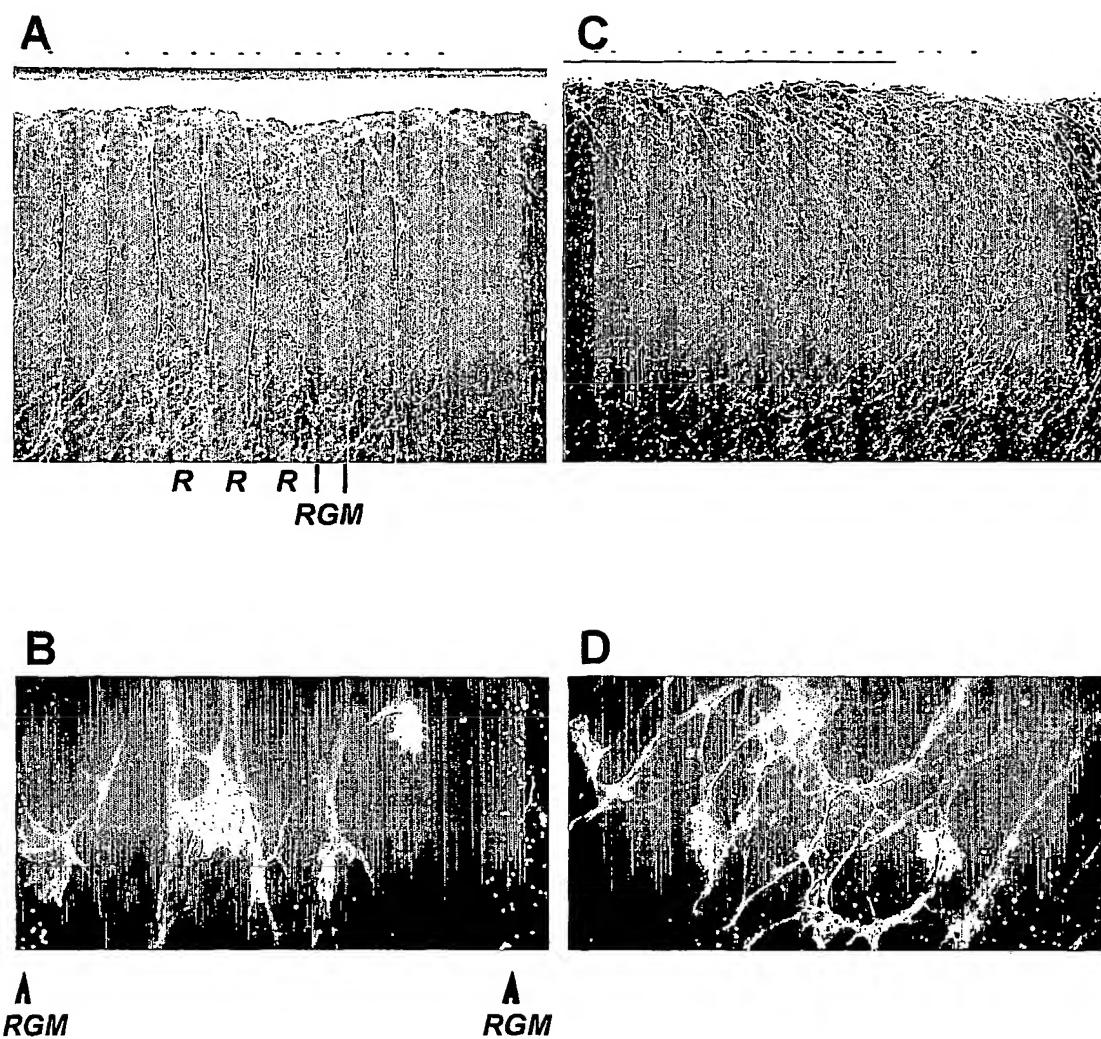


Fig.8

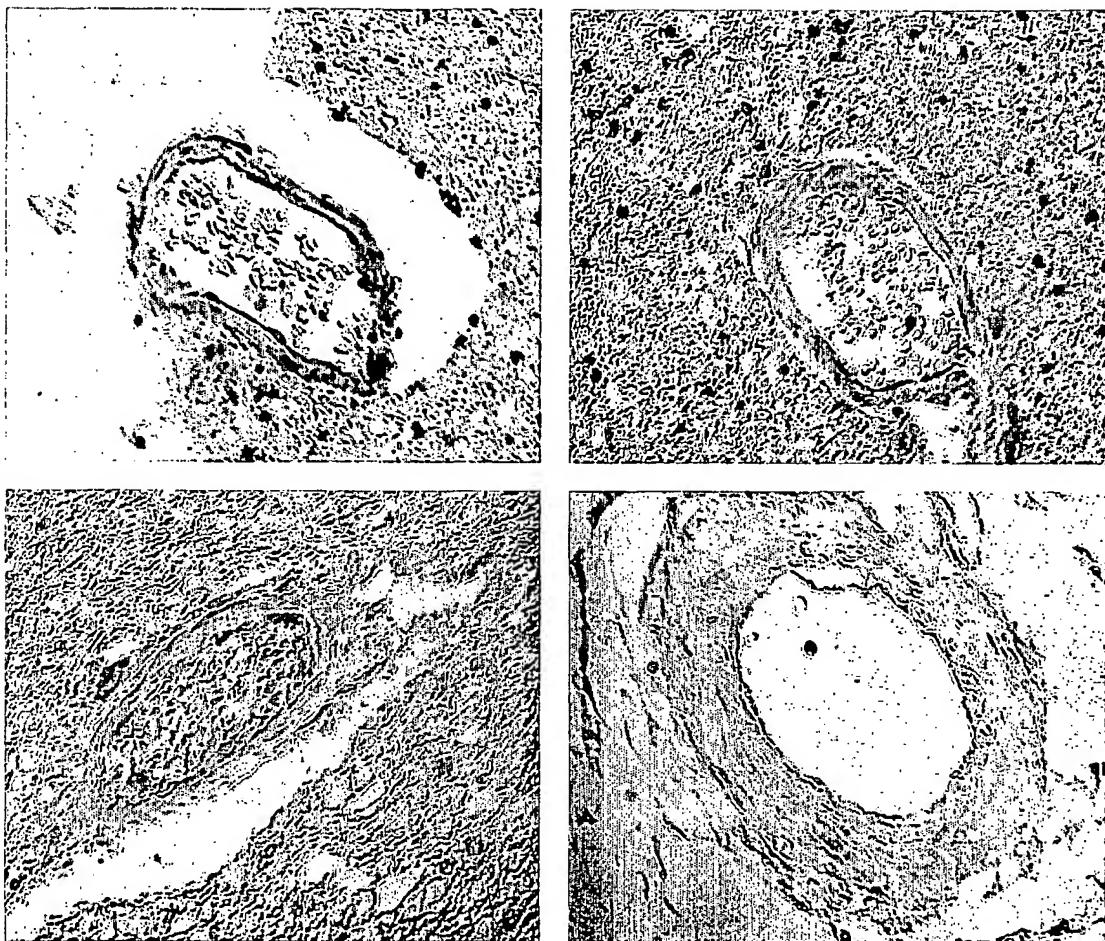


Fig.9

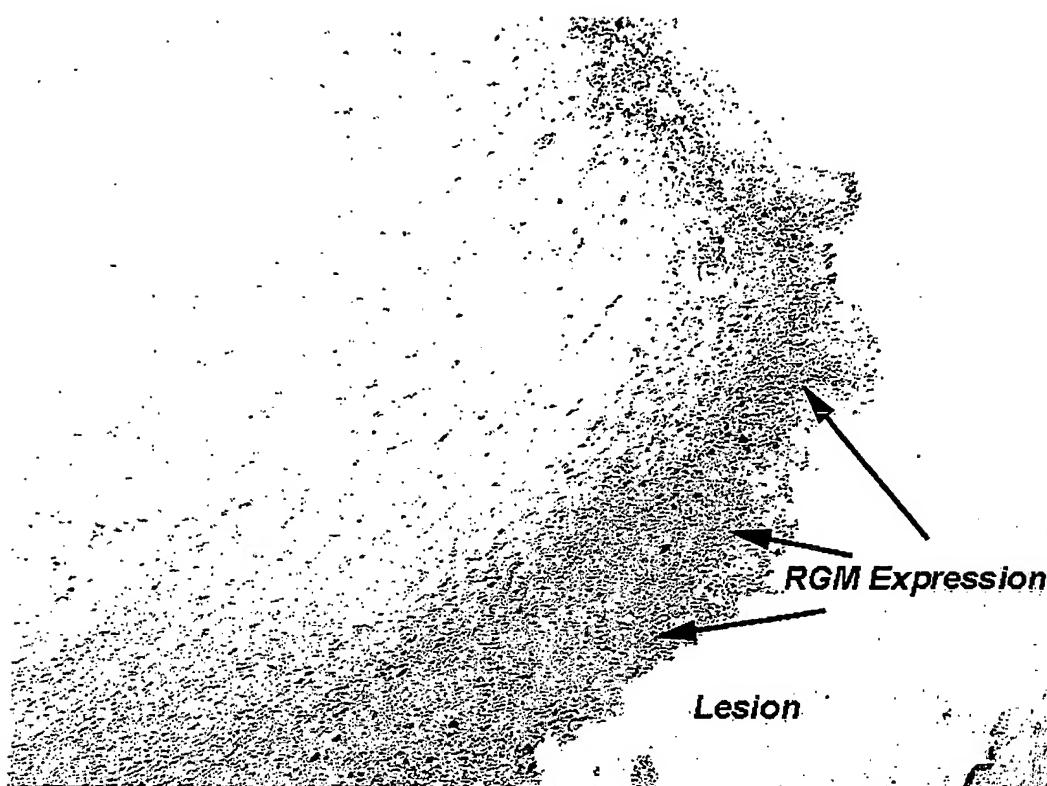
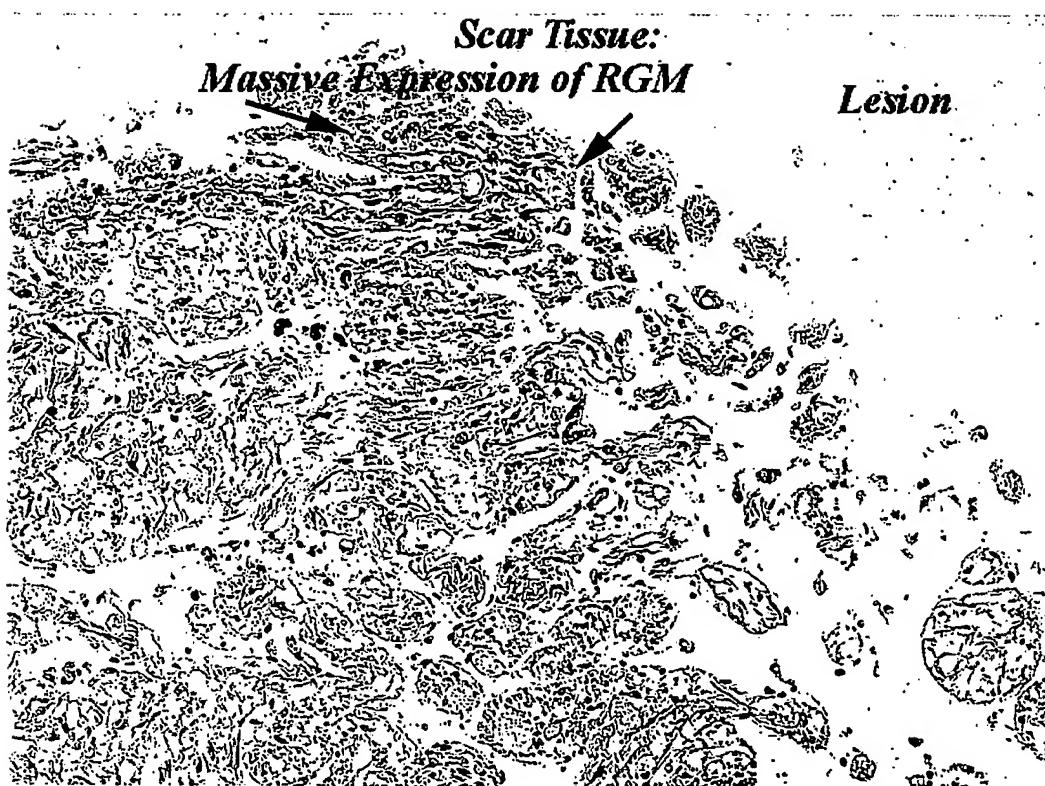


Fig.10



SEQUENCE LISTING

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<120> Use of RGM and its modulators

<130> E 2006 PCT

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<151> 2000-12-22

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Phe	Gln	Thr	Cys	Lys	Val	Gln	Gly	Ala	Trp	Pro	Leu	Ile	Asp	Asn	Asn
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Ala Lys Tyr Ile Gly Thr Thr Ile Val Val Arg Gln Val Gly Arg Tyr
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Asp Arg Asp Ser Gln Gly Leu Tyr Leu Cys Leu Arg Gly Cys Pro Leu
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Gly Arg Ala Arg Arg Lys Gly Pro Ser Leu Pro Ala Pro Pro Glu Ala
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Glu Asp Leu Tyr Phe Gln Ser Cys Val Phe Asp Leu Leu Thr Thr Gly
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Asn Ala Val Glu Asp Arg Asp Ser Gln Gly Leu Tyr Leu Cys Leu Arg
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Gly Cys Pro Leu Asn Gln Gln Ile Asp Phe Gln Thr Phe Arg Leu Ala
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Ser Tyr Ala Leu Cys Thr Arg Arg Thr Ala Arg Thr Cys Arg Gly Asp
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Asn	Phe	Gln	Thr	Cys	Lys	Val	Glu	Gly	Ala	Trp	Pro	Leu	Ile	Asp	Asn
	180					185			190						
Asn	Tyr	Leu	Ser	Val	Gln	Val	Thr	Asn	Val	Pro	Val	Val	Pro	Gly	Ser
	195					200			205						
Ser	Ala	Thr	Ala	Thr	Asn	Lys	Ile	Thr	Ile	Ile	Phe	Lys	Ala	His	His
	210					215			220						
Glu	Cys	Thr	Asp	Gln	Lys	Val	Tyr	Gln	Ala	Val	Thr	Asp	Asp	Leu	Pro
	225					230			235			240			
Ala	Ala	Phe	Val	Asp	Gly	Thr	Thr	Ser	Gly	Gly	Asp	Ser	Asp	Ala	Lys
	245					250			255						
Ser	Leu	Arg	Ile	Val	Glu	Arg	Glu	Ser	Gly	His	Tyr	Val	Glu	Met	His
	260					265			270						
Ala	Arg	Tyr	Ile	Gly	Thr	Thr	Val	Phe	Val	Arg	Gln	Val	Gly	Arg	Tyr
	275					280			285						
Leu	Thr	Leu	Ala	Ile	Arg	Met	Pro	Glu	Asp	Leu	Ala	Met	Ser	Tyr	Glu
	290					295			300						

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Glu Ser Gln Asp Leu Gln Leu Cys Val Asn Gly Cys Pro Leu Ser Glu
 305 310 315 320

Arg Ile Asp Asp Gly Gln Gly Gln Val Ser Ala Ile Leu Gly His Ser
325 . 330 335

Leu Pro Arg Thr Ser Leu Val Gln Ala Trp Pro Gly Tyr Thr Leu Glu
340 345 350

Thr Ala Asn Thr Gln Cys His Glu Lys Met Pro Val Lys Asp Ile Tyr
355 360 365

Phe Gln Ser Cys Val Phe Asp Leu Leu Thr Thr Gly Asp Ala Asn Phe
370 375 380

Thr Ala Ala Ala His Ser Ala Leu Glu Asp Val Glu Ala Leu His Pro
385 390 395 400

Arg Lys Glu Arg Trp His Ile Phe Pro Ser Ser Gly Asn Gly Thr Pro
405 410 415

Arg Gly Gly Ser Asp Leu Ser Val Ser Leu Gly Leu Thr Cys Leu Ile
420 425 430

Leu Ile Val Phe Leu
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<211> 1272
<212> DNA
<213> *Homo sapien*

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gctgagtagc	tatcgccac	tctgagccctt	agaggtgggg	gttcatcagg	agcacttcga	180
ggaggaggag	gaggaggccg	gggtggaggg	gtgggctctg	gccccctctg	tcgagccctc	240
cgctccatag	cgctctgcac	tcggcgcacc	gccccaccc	gccgcgggg	cctcgccctc	300
cattcgccgg	tacatggcat	cgaagaccc	atgatccagc	acaactgtct	ccgccaggc	360
cctacagccc	ctccccccgc	ccggggcccc	gccctccag	gcgegggctc	cggcctccct	420
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tcgattcaaa	ctgctaacc	tgggaaccat	gtggagatcc	aagctgccta	cattggcaca	840
actataatca	ttcggcagac	agctgggcag	ctctccctt	ccatcaaggt	agcagaggat	900
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agtca	gtc	atc	atc	atc	atc	1020
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gatgttttaa	tttctggta	tcccaactt	accgtggcag	ctcaggcagc	actggaggat	1140
gccccggcc	tcctggccaga	cttagagaag	ctgcatctct	tcccctcaga	tgctgggg	1200
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tgcat	aa					1272

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<210> 25
<211> 423
<212> PRT
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<400> 25
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Leu Ser Thr Leu Thr Leu Leu Leu Leu Cys Gly His Ala His Ser
20 25 30

Gln Cys Lys Ile Leu Arg Cys Asn Ala Glu Tyr Val Ser Ser Thr Leu
35 40 45

Ser Leu Arg Gly Gly Ser Ser Gly Ala Leu Arg Gly Gly Gly
50 55 60

Gly Gly Arg Gly Gly Val Gly Ser Gly Gly Leu Cys Arg Ala Leu
65 70 75 80

Arg Ser Tyr Ala Leu Cys Thr Arg Arg Thr Ala Arg Thr Cys Arg Gly
85 90 95

Asp Leu Ala Phe His Ser Ala Val His Gly Ile Glu Asp Leu Met Ile
100 105 110

Gln His Asn Cys Ser Arg Gln Gly Pro Thr Ala Pro Pro Pro Pro Arg
115 120 125

Gly Pro Ala Leu Pro Gly Ala Gly Ser Gly Leu Pro Ala Pro Asp Pro
130 135 140

Cys Asp Tyr Glu Gly Arg Phe Ser Arg Leu His Gly Arg Pro Pro Gly
145 150 155 160

Phe Leu His Cys Ala Ser Phe Gly Asp Pro His Val Arg Ser Phe His
165 170 175

His His Phe His Thr Cys Arg Val Gln Gly Ala Trp Pro Leu Leu Asp
180 185 190

Asn Asp Phe Leu Phe Val Gln Ala Thr Ser Ser Pro Met Ala Leu Gly
195 200 205

Ala Asn Ala Thr Ala Thr Arg Lys Leu Thr Ile Ile Phe Lys Asn Met
210 215 220

Gln Glu Cys Ile Asp Gln Lys Val Tyr Gln Ala Glu Val Asp Asn Leu
225 230 235 240

Pro Val Ala Phe Glu Asp Gly Ser Ile Asn Gly Gly Asp Arg Pro Gly
245 250 255

Gly Ser Ser Leu Ser Ile Gln Thr Ala Asn Pro Gly Asn His Val Glu
260 265 270

Ile Gln Ala Ala Tyr Ile Gly Thr Thr Ile Ile Ile Arg Gln Thr Ala
275 280 285

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Gly Gln Leu Ser Phe Ser Ile Lys Val Ala Glu Asp Val Ala Met Ala
290 295 300

Phe Ser Ala Glu Gln Asp Leu Gln Leu Cys Val Gly Gly Cys Pro Pro
305 310 315 320

Ser Gln Arg Leu Ser Arg Ser Glu Arg Asn Arg Arg Gly Ala Ile Thr
325 330 335

Ile Asp Thr Ala Arg Arg Leu Cys Lys Glu Gly Leu Pro Val Glu Asp
340 345 350

Ala Tyr Phe His Ser Cys Val Phe Asp Val Leu Ile Ser Gly Asp Pro
355 360 365

Asn Phe Thr Val Ala Ala Gln Ala Ala Leu Glu Asp Ala Arg Ala Phe
370 375 380

Leu Pro Asp Leu Glu Lys Leu His Leu Phe Pro Ser Asp Ala Gly Val
385 390 395 400

Pro Leu Ser Ser Ala Thr Leu Leu Ala Pro Leu Leu Ser Gly Leu Phe
405 410 415

Val Leu Trp Leu Cys Ile Gln
420